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PHYSIOLOGICAL EFFECTS OF CARBOHYDRATES
IN THE NUTRITION OF A MOSQUITO, *AEDES*
AEGYPTI AND TWO FLIES, *SARCOPHAGA*
BULLATA AND *MUSCA DOMESTICA*

RACHEL GALUN¹ AND G. FRAENKEL

Department of Entomology, University of Illinois, Urbana

ONE FIGURE

Many adult dipterous, hymenopterous and lepidopterous insects attain their maximum lifespan if kept on aqueous sugar solutions only. Several workers have used the lifespan of insects as a criterion for the nutritional value of the carbohydrate on which the insect was kept. A sugar that in its aqueous solution did not prolong the survival of an insect beyond that on water alone was regarded as non-nutritive for this insect.

It also emerged from these studies that the ingestion of carbohydrates depended on their relative sweetness for the insect, and that some sugars even appeared to be repelling (Hassett, '48). The survival could, therefore, be a function of the sweetness as well as the nutritive value of the carbohydrate. Attempts have been made to overcome this difficulty by offering the carbohydrate solution as the sole water source (Fraenkel, '40) or by adding small amounts of sucrose to the solutions thereby rendering them more palatable (Vogel, '31; Hasslinger, '35; Hassett, '48). In no case was the rate

¹In partial fulfillment of the requirements for the Ph.D. in entomology at the University of Illinois. Present address: Israeli Institute for Biological Research, Ness Ziona, Israel.

of ingestion measured, and it still remains doubtful whether any of these methods fully compensate for the absence of sweetness or an existing repellency.

In recent experiments one of us (Fraenkel, '55) attempted to classify the non-nutritive carbohydrates into inert and inhibitory compounds for the growth of *Tenebrio molitor* L. larvae. A sugar was considered inert if its mixture with starch gave the same growth rate as a corresponding starch-cellulose or starch-glass powder mixture. Carbohydrate-starch mixtures showing a lower growth rate than the corresponding glass powder or cellulose mixtures were considered inhibitory. The possibility that a carbohydrate-starch mixture might have been consumed in smaller amounts than the starch-cellulose or glass powder mixture, due to some repellent effect of the carbohydrate, was disregarded.

In the present work the utilization of 28 carbohydrates by the adults of *Aedes aegypti* L., *Sarcophaga bullata* Park. and *Musca domestica* L. was studied. The findings were correlated with the presence of the corresponding digestive enzymes in the adults and other developmental stages of these three insects. An attempt was then made to classify carbohydrates which did not show nutritional values into toxic, repelling and inert materials by measuring the actual amounts ingested in each instance.

MATERIALS AND METHODS

Materials used. The following compounds were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio: d(—) ribose, l(+) arabinose, l(+) rhamnose, d(+) mannose, l(—) sorbose, d(+) maltose, d(+) trehalose, d(+) cellobiose, d(+) melezitose, soluble starch, glycogen, α -methyl-d-glucoside, α -methyl-d-mannoside, d-sorbitol, dulcitol, and i-inositol.

Pfanstiehl Chemical Company, Waukegan, Illinois, supplied: d(+) glucose, d(—) fructose, d(+) galactose, inulin, and d(—) mannitol.

d(+) Xylose, d(+) melibiose, and d(+) raffinose were obtained from Difco Laboratories, Inc., Detroit, Michigan.

Sucrose, d(+) lactose, and dextrin were supplied by Allied Chemicals and Dye Corporation, Merck and Co., Inc., and the Coleman and Bell Co. respectively.

Survival experiments. Newly emerged adults were maintained on 5% solutions of the test carbohydrates in water, offered *ad libitum* and changed daily. The cages measuring $9 \times 9 \times 9$ inches were kept at $28 \pm 2^\circ\text{C}$. and under constant illumination throughout the experiment. The mortality was recorded daily.

Enzyme tests. The presence of carbohydrates in the gut was tested *in vitro* with oligosaccharides, polysaccharides and glycosides as substrates. These experiments were carried out on larvae, pupae and adults of all three insects. Larvae were used while still in the feeding stage. In order to minimize errors resulting from intestinal contamination, the insects were starved for 24 hours prior to testing. Tests on the pupae were conducted at the following intervals: house fly, daily; *Sarcophaga*, every other day; *Aedes*, on the second day after pupation. Adults were used within 24 hours after emergence. They were starved until used, in order to minimize growth of intestinal bacteria which may take part in digestion.

Enzyme preparations were made from the guts of larvae and adult flies, or from whole pupae, excluding the puparia. In the case of mosquitoes the whole insects were homogenized. The guts were dissected out, ground up in wet sand and the resulting suspension was distributed among small test tubes ($2'' \times \frac{1}{3}''$) so that each tube contained the equivalent of about one-half of a gut (or 5 insects in the case of mosquitoes).

The experimental protocol was as follows: 0.5 ml of a 5% solution of the carbohydrate to be tested and one drop of toluene (to prevent bacterial growth) were added to each test tube containing the homogenate described above, and the mixtures were incubated for 24 hours at 28°C . Using the osazone test, the appearance of glucosazone crystals was taken as the criterion for enzyme action. Controls were run with gut suspension in which the enzymes had been destroyed by boiling.

Toxicity experiments. All the carbohydrates that failed to promote survival were tested for toxic properties by employing solutions containing five grams of sucrose and five grams of the compound in question in 100 ml of water. Any decrease in the survival time as compared to the survival on a 5% sucrose solution was interpreted as a toxic effect. Fresh solutions were prepared every week and kept under refrigeration.

Additional toxicity experiments were carried out on house flies, using a method (Dethier and Rhoades, '54) which permits quantitative measurement of fluid intake and lethal dose: A J shaped 5 ml. volumetric pipette was filled with the test solution and offered to a group of 20 flies in a mason jar. Measurement of the amount of fluid removed by the feeding insects from the tip of the pipette was accomplished by means of a 1 ml calibrated syringe which was inserted into the capillary opening of the pipette. Water was forced out of the syringe until the meniscus in the pipette returned to the original mark. The amount of the fluid removed from the pipette was thus read directly from the syringe. Controls for evaporation were set up in each experiment and the losses subtracted from the readings.

This procedure was repeated for 4 24-hour periods in succession for each carbohydrate. Three jars were prepared for each solution to be studied so that ingestion per fly per day was in all cases the average of 240 readings.

Test for repellent properties. Carbohydrates which in combination with 5% sucrose were consumed in amounts smaller than 5% sucrose alone were further tested for their repelling properties, using ingestion measurements as described above. Two pipettes were placed in each jar, one containing a 5% sucrose solution, and the other a solution of 5 gm of sucrose and 5 gm of the test carbohydrate per 100 ml water. The consumption of the two solutions were measured according to the procedure described above. The significance of the difference between the mean consumption of the two solutions was determined by Fisher's "t" test.

RESULTS AND DISCUSSION

Feeding experiments

The results of the feeding experiments are shown in table 1, which states the numbers of days to 50 and 100% mortality on various carbohydrates. Table 2 summarizes the results by comparison with the nutritive value of sucrose which is taken as unity. Nutritive value (n.v.) is then:

$$\text{n.v.} = \frac{\text{Survival on test sugar} - \text{survival on water}}{\text{Survival on sucrose} - \text{survival on water}}$$

The values for 50% mortality were used.

In general, the insects survived for long periods on glucose, fructose, sucrose, maltose, raffinose, melezitose, and sorbitol, and failed to survive on xylose, ribose, arabinose, rhamnose, sorbose, cellobiose, inulin, α -methylmannoside, dulcitol and inositol. There were great differences, between insect species, in the survival value of mannose, galactose, trehalose, melibiose, lactose, dextrin, starch, glycogen, α -methylglucoside, and mannitol (tables 1 and 2).

The findings to some extent correspond to the feeding habits of the three insects. Adult mosquitoes derive their carbohydrates mostly from nectar, a material containing a limited number of sugars including fructose, glucose, sucrose, maltose, raffinose and melibiose. The house fly on the other hand, is quite omnivorous, feeding on a variety of materials which among other carbohydrates may contain starch, glycogen and lactose. The cockroach which has similar omnivorous feeding habits, has also been shown to utilize starch and lactose (Noland, Lilly and Baumann, '49).

Sarcophaga which appears to occupy an intermediate position feeds on nectar and unlike the mosquito, is also attracted by putrified meat, which serves as a source of protein necessary for egg production, and may contain glucogen.

The nutritional value of α -methylglucoside was found to be 0.5 for *Sarcophaga* but only 0.3 for the house fly. Tests revealed, in both insects, enzymes that split this glycoside. It was suspected that methanol, a likely product of this

TABLE 1

Survival of the mosquito Aedes aegypti (♀) and the flies Sarcophaga bullata and Musca domestica fed on solutions of various carbohydrates

| COMPOUND | AEDES AEGYPTI | | | SARCOPHAGA BULLATA | | | MUSCA DOMESTICA | | |
|--------------------|---------------|-------------------------|------|--------------------|-------------------------|------|-----------------|-------------------------|------|
| | No. used | Days to mortality of | | No. used | Days to mortality of | | No. used | Days to mortality of | |
| | | 50% | 100% | | 50% | 100% | | 50% | 100% |
| Water | 44 | 5.5-8 | 7-11 | 25 | 3.8 | 5 | 123 | 1-2 | 2-3 |
| Pentoses | | | | | | | | | |
| Xylose | 32 | 6.3 | 11 | 23 | 3.2 | 4 | 38 | 1-2 | 2 |
| Ribose | 27 | 6.0 | 10 | 24 | 5.0 | 9 | 130 | 2.5 | 4 |
| Arabinose | 33 | 5.2 | 6 | 27 | 2.8 | 4 | 59 | 1 | 2 |
| Rhamnose | 31 | 6.0 | 9 | 36 | 3.8 | 6 | 74 | 2.5 | 4 |
| Hexoses | | | | | | | | | |
| Glucose | 20 | 27 | 67 | 37 | 19 | 42 | 80 | 17 | 24 |
| Fructose | 20 | 33 | 40 | 34 | 17 | 38 | 76 | 13 | 20 |
| Mannose | 22 | 6.2 | 8 | 30 | 19 | 34 | 48 | 25 | 46 |
| Galactose | 20 | 14.5 | 22 | 23 | 16 | 39 | 44 | 20 | 31 |
| Sorbose | 20 | 4.2 | 8 | 44 | 5.5 | 7 | 46 | 2 | 3 |
| Disaccharides | | | | | | | | | |
| Sucrose | 21 | 32 | 67 | 23 | 21 | 34 | 26 | 20 | 31 |
| Maltose | 20 | 26 | 64 | 23 | 16 | 40 | 42 | 15 | 22 |
| Trehalose | 23 | 12 | 35 | 55 | 14 | 29 | 36 | 14 | 26 |
| Melibiose | 30 | 12 | 36 | 26 | 11 | 20 | 68 | 17 | 22 |
| Lactose | 20 | 7.3 | 10 | 21 | 4.5 | 8 | 42 | 17 | 32 |
| Cellobiose | 28 | 5.5 | 7 | 25 | 3.5 | 5 | 56 | 1.5 | 3 |
| Trisaccharides | | | | | | | | | |
| Raffinose | 20 | 17 | 39 | 54 | 18 | 40 | 43 | 14 | 22 |
| Melzitose | 20 | 29 | 57 | 23 | 14 | 23 | 42 | 15 | 25 |
| Polysaccharides | | | | | | | | | |
| Dextrin | 32 | 10 | 21 | 31 | 19 | 35 | 25 | 16 | 26 |
| Starch | 24 | 4 | 8 | 35 | 9 | 24 | 31 | 18 | 27 |
| Glycogen | 26 | 5.5 | 9 | 35 | 6 | 10 | 75 | 11 | 16 |
| Inulin | 28 | 3.5 | 6 | 22 | 3 | 5 | 74 | 2 | 3 |
| Glycosides | | | | | | | | | |
| α-methyl-glucoside | 23 | 4 | 8 | 40 | 12 | 34 | 37 | 8 | 16 |
| α-methyl-mannoside | 32 | 2 | 6 | 35 | 2.8 | 6 | 61 | 1.5 | 3 |
| Sugar alcohols | | | | | | | | | |
| Glycerol | 20 | 3 | 7 | 30 | 6.5 | 10 | 55 | 3.5 | 7 |
| Mannitol | 34 | 4 | 6 | 61 | 15 | 33 | 88 | 17 | 22 |
| Sorbitol | 22 | 15 | 63 | 27 | 16 | 32 | 41 | 11 | 15 |
| Dulcitol | 28 | 3 | 6 | 25 | 2.5 | 4 | 41 | 2 | 2 |
| Inositol | 33 | 3 | 6 | 32 | 6 | 9 | 40 | 3 | 5 |

cleavage, may accumulate and poison the house fly. A group of 20 flies was kept on a 5% α -methylglucoside solution while a similar control group was kept on a solution containing only the products of hydrolysis of the test solution (a 5% glucose solution containing one ml of methanol per 100 ml). The results are presented in figure 1. The consumption of the two solutions by the flies was about equal, but the mortality on the

TABLE 2

The nutritive values of various carbohydrates for Aedes aegypti, Sarcophaga bullata and Musca domestica, calculated in the following manner:

$$\text{Nutritional value} = \frac{\text{survival on test carbohydrate} - \text{survival on water}}{\text{survival on sucrose} - \text{survival on water}}$$

| COMPOUND | A. AEGYPTI | S. BULLATA | M. DOMESTICA | COMPOUND | A. AEGYPTI | S. BULLATA | M. DOMESTICA |
|------------|---------------|---------------|-----------------|--------------------------------|---------------|---------------|-----------------|
| Arabinose | 0.0 | 0.0 | 0.0 | Raffinose | 0.5 | 0.9 | 0.7 |
| Xylose | 0.0 | 0.1 | 0.0 | Melezitose | 0.9 | 0.6 | 0.7 |
| Ribose | 0.0 | 0.0 | 0.0 | Dextrin | 0.2 | 0.9 | 0.8 |
| Rhamnose | 0.0 | 0.0 | 0.0 | Starch | 0.0 | 0.3 | 0.9 |
| Glucose | 0.8 | 0.9 | 0.8 | Glycogen | 0.0 | 0.1 | 0.5 |
| Fructose | 1.0 | 0.8 | 0.6 | Inulin | 0.0 | 0.0 | 0.0 |
| Mannose | 0.0 | 0.9 | 1.3 | α -methyl- glycoside | 0.0 | 0.5 | 0.3 |
| Galactose | 0.3 | 0.7 | 1.0 | α -methyl- mannoside | 0.0 | 0.0 | 0.0 |
| Sorbose | 0.0 | 0.1 | 0.0 | Glycerol | 0.0 | 0.1 | 0.1 |
| Sucrose | 1.0 | 1.0 | 1.0 | Mannitol | 0.0 | 0.7 | 0.0 |
| Maltose | 0.8 | 0.8 | 0.7 | Sorbitol | 0.5 | 0.7 | 0.8 |
| Trehalose | 0.3 | 0.7 | 0.7 | Dulcitol | 0.0 | 0.0 | 0.0 |
| Melibiose | 0.3 | 0.4 | 0.8 | Inositol | 0.0 | 0.1 | 0.1 |
| Lactose | 0.0 | 0.0 | 0.8 | | | | |
| Cellobiose | 0.0 | 0.0 | 0.0 | | | | |

α -methylglucoside solution was much higher. None of the flies kept on the glucose-methanol solution died within the duration of the experiment. It is tentatively concluded that the mortality on α -methylglucoside is not due to the production of methyl alcohol in the process of digestion. The question, however, remains open as to whether the methyl alcohol fed as such reaches the same site as and is identical with the products of enzymatic cleavage.

The low nutritional value of the glycoside as compared to glucose may also have its source in a slow rate of hydrolysis thereby yielding too little glucose for adequate nourishment. It is also possible that the glycoside *per se* is toxic and that the unhydrolysed portions of it affect both *M. domestica* and *S. bullata*. This suggestion is supported by the fact that the glycoside is highly toxic to *A. aegypti* which were found not

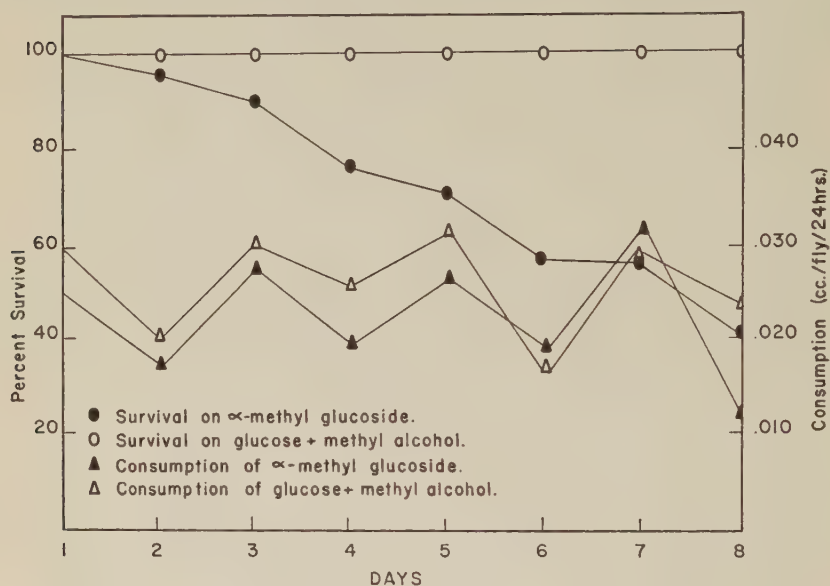


Fig. 1 Survival of *Musca domestica* (adult) on α -methylglucoside ●—●—● and on an equivalent mixture of glucose and methyl alcohol ○—○—○. The figure also contains data about the consumption of solutions of α -methylglucoside ▲—▲—▲ and of a mixture of glucose and methyl alcohol △—△—△ per fly during the same period.

to possess the proper hydrolytic enzyme. α -Methylglucoside was also found to be inhibitory for *Tenebrio molitor* (Fraenkel, '55).

Enzyme tests

A comparison of the digestive enzymes found in the three adult insects (as summarized in table 3) with the nutritional values of the oligosaccharides, polysaccharides and glycosides

(table 2), indicates that only those compounds for which the corresponding hydrolytic enzymes are present in the adult gut are of any nutritional value. The two exceptions to this were melibiose, which had some nutritional value for all three insects, and lactose which was utilized by the house fly. In neither case was the presence of the appropriate enzyme demonstrated.

Since there was a possibility that the conditions used for the tests were not generally favorable for establishing the presence of the enzymes in question, a number of modifications of the original procedure were attempted. All the previous tests had been carried out in unbuffered solutions, so a series of buffered substrates were made up at the following pH's: 3.6, 4.4, 5.3, 6.2, 7.0 and 7.7. These were tested with the homogenates in the usual manner. The results with lactose and melibiose substrates were again negative.

It is well known that the rate of activity of certain enzymes diminishes during starvation. Since the enzyme tests were usually performed on tissues from previously starved insects, the chance that the enzymes were being lost in this process required investigation. Consequently the insects were fed on either melibiose or lactose previous to preparing the homogenates. Neither enzyme was found to be present.

Intestinal microorganisms are of importance in the breakdown of some food constituents in many insects. In order to investigate the possible role of such microorganisms in the house fly, a sterile culture was obtained for the enzyme tests. The following procedure was adopted for this purpose: A 125 ml conical flask containing fermented CSMA² mixture was plugged with a cotton wad and autoclaved for 30 minutes. A batch of fly eggs which had been sterilized for 20 minutes in a 1% Zephiran chloride solution (Winthrop-Stearns Inc.) was inoculated into this medium using the method described by Brust and Fraenkel ('55). The insects were left on this

² A mixture of 33.3% wheat bran, 26.7% alfalfa meal and 40% brewers grain (obtained from the Ralston-Purina Co., St. Louis, Mo.) moistened with a yeast suspension and solution of malt extract.

medium until the completion of development, and the adults were tested for carbohydrates upon emergence. The results were in all cases identical with those obtained from non-sterile cultures.

The possibility that in spite of the technique employed the house fly harbors microorganisms which were carried in the egg is remote, since Brookes (unpublished) found the medium of cultures so treated to remain sterile throughout the development of the larvae.

Enzymatic changes throughout development

The enzymatic patterns of the insects in question are markedly different in their various stages of development (table 3). Homogenates of mosquitoes larvae *in vitro* hydrolysed sucrose, maltose, trehalose, melezitose, dextrin, starch and glycogen. Homogenates of adults no longer hydrolysed starch and glycogen but contained an enzyme which splits raffinose. Such changes are even more pronounced in *Sarcophaga* and the house fly. Only invertase was detected in homogenates from larval intestine but those of adults of both species possessed enzymes for the cleavage of sucrose, maltose, trehalose, melezitose, raffinose, dextrin, starch, glycogen and α -methylglucoside (table 3).

A gradual acquisition of carbohydrates was demonstrated during pupal development. In the house fly, for example, very few enzymes could be distinguished on the first day after the formation of the puparium. By the end of the second day, however, all the carbohydrases of the adult had made their appearance. These changes can be correlated with some of the major morphological changes of pupal development, such as evagination of the head and legs which also take place on the second day. The same essentially holds for *Sarcophaga*. Here, melezitase could be demonstrated on the 5th day and raffinase on the 7th day.

The development of *Sarcophaga* on synthetic diets has never been studied. It is however well known for the larvae of other fleshflies that they develop normally in the absence

of carbohydrates and are lacking in carbohydrases. The larvae of *Phormia regina* on a synthetic and sterile diet and in the absence of a carbohydrate reach the same weight as on a natural diet (Brust and Fraenkel, '55). Moreover, the growth of *Phormia* is retarded by replacing 10% of the casein in their diet with glucose (McGinnis *et al.*, '55). A lack of carbohydrases has been demonstrated in the larvae of another blowfly, *Lucilia serricata* (Hobson, '31). Similarly, the housefly larva on a sterile synthetic diet develops well in the absence of a carbohydrate (Brookes, unpublished).

The effect of carbohydrates on the development of *Aedes aegypti* larvae has never been investigated. Synthetic diets for this insect, prepared for other nutritional studies have always contained some sugars (Trager, '48; Golberg and DeMeillon, '48), although their necessity has never been demonstrated. Wigglesworth's ('48) studies on starved larvae have indicated that a large number of carbohydrates can serve as glycogen precursors, and probably postpone death from starvation of larvae deprived of proteins. Still, these observations do not prove the necessity of carbohydrates for larvae raised in the presence of proteins. The presence of carbohydrases in *A. aegypti* larvae is in good agreement with their ability to convert carbohydrates to glycogen, as demonstrated by Wigglesworth.

Toxicity experiments

The carbohydrates which failed to promote survival of the insects in straight feeding trials, were tested again incorporated in a 5% sucrose solution which by itself ensures optimal survival. In almost every instance, the admixture of a non-nutritive sugar decreased the survival time. This was considered as to indicate toxic effects of the non-nutritive sugars (tables 4 and 5).

With *Aedes aegypti*, the most striking effects of reduction of survival were given by ribose, arabinose, rhamnose, sorbose, α -methylmannoside, α -methylglucoside and glycerol, and lesser

effects by xylose, mannitol, dulcitol, inositol and possibly mannose.

An unexplained inconsistency concerns the effect of glycerol and α -methylglucoside. These compounds both were seemingly very toxic at the 50% level, but some of the experimental insects were much more resistant to these substances than others, so that complete mortality did not occur until more than 24 days after the initiation of the feeding.

TABLE 4

Apparent toxicity of various carbohydrates for Aedes aegypti and Sarcophaga bullata. Concentrations of all solutions: 5 gm of a carbohydrate plus 5 gm sucrose per 100 ml water. Insects fed ad libitum on cotton soaked in solutions, with change of solution daily.

| | AEDES AEGYPTI | | | SARCOPHAGA BULLATA | | |
|---------------------------|---------------|-----------------------|------------------------|--------------------|-----------------------|------------------------|
| | Number used | Days to 50% mortality | Days to 100% mortality | Number used | Days to 50% mortality | Days to 100% mortality |
| Sucrose (control) | 21 | 32 | 67 | 23 | 21 | 34 |
| Arabinose | 23 | 5 | 14 | 51 | 9 | 13 |
| Xylose | 24 | 13 | 25 | 40 | 18 | 23 |
| Ribose | 21 | 7.5 | 12 | 23 | 11 | 20 |
| Rhamnose | 27 | 4 | 11 | 28 | 10 | 17 |
| Mannose | 29 | 24 | > 31 | | | |
| Sorbose | 34 | 3 | 12 | 35 | 7 | 16 |
| α -Methylglucoside | 33 | 6 | > 24 | | | |
| α -Methylmannoside | 33 | 2 | 6 | 43 | 3.5 | 11 |
| Mannitol | 32 | 14 | 34 | | | |
| Dulcitol | 28 | 10 | 25 | 37 | 5 | 13 |
| Inositol | 26 | 12 | 23 | 71 | 17 | 28 |
| Glycerol | 31 | 6 | > 27 | | | |

Mannose which is not utilized by the mosquito at all, was not toxic when fed in a mixture with 5% sucrose. However, since investigations by Staudenmeyer with bees ('39) had demonstrated that the toxicity of mannose increases as the concentration of sucrose or glucose in the medium is decreased, similar experiments were performed with *Aedes*. The mosquitoes were offered a mixture of 1 gm of glucose and 5 gm of mannose in 100 ml of water and mortality was assessed by daily observation. It was found that 50% mortality oc-

curred only after 18 days, and some mosquitoes survived as long as 35 days. From this evidence it was concluded that mannose is not toxic for mosquitoes.

Sarcophaga bullata. The following carbohydrates clearly reduced the survival time, in order of decreasing toxicity (table 4): α -methylmannoside, dulcitol, sorbose, arabinose,

TABLE 5

Consumption and toxicity of various carbohydrate solutions by and for Musca domestica. Sixty flies were used in each test

| COMPOUND | CONCENTRATION 5% IN WATER | CONCENTRATION: 5% IN WATER PLUS 5% SUCROSE ADDED TO EACH SOLUTION | | |
|---------------------------|---|--|---|-----------------------------|
| | CONSUMPTION CM ³ /FLY/24 HRS. | Days to 50 % mortality | Consumption cm ³ /fly/24 hrs. | LD50 ¹ mg/fly |
| Water (control) | .024 | | | |
| Arabinose | .015 | 4 | .011 | 2.15 |
| Xylose | .009 | 6 | .014 | 4.10 |
| Ribose | | 7 | .018 | 6.25 |
| Rhamnose | | 4 | .017 | 2.40 |
| Glucose | .030 | | | |
| Fructose | .032 | | | |
| Galactose | .033 | | | |
| Mannose | .021 | | | |
| Sorbose | .011 | 4 | .020 | 3.90 |
| Sucrose | .029 | 20 | .029 | non toxic |
| Maltose | .029 | | | |
| Melibiose | .024 | | | |
| Lactose | .024 | | | |
| α -Methylmannoside | | 3 | .015 | 2.30 |
| Dulcitol | | > 10 | .025 | non toxic |
| Inositol | | 7 | .019 | 7.00 |

¹ LD50 = quantity of carbohydrate (calculated as milligrams per fly) necessary to decrease population to 50%.

rhamnose, and ribose. In general, this insect, of the three studied, seems to be the most resistant to toxic factors.

Musca domestica. The results obtained with this fly are summarized in table 5. On sucrose alone, 50% of the flies had died after 20 days, while on mixtures of sucrose with a number of "toxic" carbohydrates, xylose, ribose, arabinose, rhamnose, sorbose, α -methylmannoside or inositol survival was reduced to between three and 7 days. Of the non-nutritive

sugars dulcitol alone did not seem to depress survival on 5% sucrose solutions to an appreciable extent. This indicated that dulcitol is inert for the housefly, a conclusion corroborated in tests to be described later.

Taste-toxicity relationships

Tables 1 and 2 show clearly that with many of the carbohydrates tested survival is about the same as on water alone. This rises the question of whether the apparent lack of nutritional value of a sugar may not be simply due to the absence of feeding. Consequently, the intake of solutions by a known number of houseflies over a definite period of time was measured using the method recently developed by Dethier and Rhoades ('54) (briefly described on page 4). The results of a number of such tests are summarized in table 5.

When various carbohydrates were fed in 5% solutions, consumption of fluid was of a similar order with all nutritive sugars, and differed only little on water alone. However, with the non-nutritive sugars arabinose, xylose and sorbose, the food intake was greatly reduced.

Similar tests were carried out with mixtures of various non-nutritive carbohydrates with sucrose. It became apparent that in almost every case, where the presence of such a sugar had greatly reduced the survival period, much less food was taken than with sucrose alone. This method allowed to express by a method, widely used in toxicological studies, the effect of a sugar as the lethal dose for 50% mortality (L.D.50) which indicates the weight of material in milligram per fly that causes a 50% mortality in a population feeding on it (table 5). The LD50 would give an accurate expression of the toxicity of each sugar if the food intake had been the same in every test solution. Since the food intakes vary considerably the possibility remains that early death of flies fed on a particular carbohydrate alone or in mixture with sucrose, was caused rather by diminished intake, due to bad

taste, than by an innate toxicity of the sugar. Several arguments may be brought to bear against this interpretation:

1. Mannose alone is consumed in similar quantities as sorbose or inositol mixed with sucrose. Yet, the flies survive for 25 days on mannose (table 1) and only 4 or 7 days on the sorbose- or inositol-sugar mixtures. Since the nutritional value of mannose and sucrose is of the same order, the bad effects of sorbose and inositol must be caused by toxicity.

2. There appears to be very little correlation between the amounts of the mixtures consumed per fly and the time required for 50% mortality (table 5). Some of the mixtures were consumed in higher quantities than others on which survival was longer. This indicates that survival is not only dependent on the quantities of fluid, viz. sucrose, taken in, but also on specific toxic effects of the admixed constituents.

3. Of all the carbohydrate-sucrose mixtures tested (table 5) consumption was lowest with arabinose. In order to test, whether and to what extent the low survival in the presence of arabinose was due to diminished feeding or to toxicity, a set of experiments was designed in which the proportions of arabinose to sucrose were changed, in one case from 5 gm arabinose to 3 gm/100 ml, with sucrose remaining at 5 gm, and the other with sucrose increased from 5 gm to 15 gm, and arabinose remaining at 5 gm (table 6). Raising the sucrose level three times lengthened the 50% mortality period only from 4 to 6 days, although the average intake of sucrose was little below that on sucrose alone (1.05 vs. 1.35 mg/fly/24 hours). In spite of the fact that the total sucrose intake was greatly changed with changing concentrations of sucrose and arabinose, the LD50 remained the same in all three cases. We may therefore conclude that the high mortalities encountered were due to the toxic effect of arabinose rather than the insufficient intake of sucrose.

The possibility that at least part of the toxicity of arabinose (and perhaps other pentoses too) was due to impurities cannot be disregarded. In a series of 8 tests with houseflies

the LD50 of arabinose was found to be 2.13 ± 0.07 (standard deviation) although different concentrations of arabinose and sucrose were used. The same preparation, after several crystallizations from glacial acetic acid and ethanol showed a diminished toxicity of LD50—3.2. The survival time was however prolonged by only one day and was still very low (table 6). This would suggest that impurities in the arabinose

TABLE 6

Response of Musca domestica to Arabinose. Sixty flies were used in each test

| COMPOUND GM/100 ML WATER | TIME TO 50% MORTALITY | CONSUMPTION | | | LD50 MG/FLY |
|--|--------------------------|-----------------------------|----------------------------|------------------------------|----------------|
| | | ml solution/ fly/24 hrs. | mg sucrose/ fly/24 hrs. | mg arabinose/ fly/24 hrs. | |
| Water | 2 | .024 | ... | .. | ... |
| Sucrose 5 | 20 | .029 | 1.35 | .. | ... |
| Arabinose 5 | 1 | .015 | ... | .75 | ... |
| Sucrose 5 Arabinose 5 | 4 | .011 | .55 | .55 | 2.15 |
| Sucrose 5 Arabinose 5 (purified) | 5 | .013 | .65 | .63 | 3.2 |
| Sucrose 5 Arabinose 3 | 5 | .014 | .70 | .42 | 2.2 |
| Sucrose 15 Arabinose 5 | 6 | .007 | 1.05 | .35 | 2.0 |

used might have decreased the survival time, without accounting wholly for the phenomenon of toxicity.

Taste — Consumption relationships

From the result so far reported it is clear that high mortality of the insects investigated on many carbohydrates cannot be due alone to low intake, that is bad taste, and must be attributed to specific toxic effect. This, however, does not eliminate the possibility, that gustatory repellency may be

a major reason for lower consumption of many toxic sugar solutions.

Dethier and Rhoades ('54) have shown that when the blowfly, *Phormia regina* Meigen, is confronted with two concentrations of glucose or sucrose, the higher of the two is always consumed in greater quantity. This has been con-

TABLE 7
Feeding preferences of Musca domestica
Solutions I and II presented simultaneously in identical pipettes

| SOLUTION CONTAINS GM/100 ML WATER I | SOLUTION CONTAINS GM/100 ML WATER II | QUANTITY CONSUMED (ml/fly/24 hrs.) | | SIGNIFICANCE OF DIFFERENCE P = 1% |
|--|--|---------------------------------------|------|---|
| | | I | II | |
| 1. Sucrose 5 | Water | .030 | .001 | + |
| 2. Sucrose 5 | Sucrose 10 | .005 | .014 | + |
| 3. Sucrose 5 | Sucrose 5 | .027 | .001 | + |
| | Xylose 5 | | | |
| 4. Sucrose 5 | Sucrose 5 | .026 | .002 | + |
| | Ribose 5 | | | |
| 5. Sucrose 5 | Sucrose 5 | .019 | .004 | + |
| | Arabinose 5 | | | |
| 6. Sucrose 5 | Sucrose 5 | .018 | .003 | + |
| | Rhamnose | | | |
| 7. Sucrose 5 | Sucrose 5 | .027 | .001 | + |
| | Sorbose 5 | | | |
| 8. Sucrose 5 | Sucrose 5 | .016 | .005 | + |
| | α -methyl- mannoside 5 | | | |
| 9. Sucrose 5 | Sucrose | .014 | .017 | — |
| | Dulcitol 5 | | | |
| 10. Sucrose 5 | Sucrose 5 | .018 | .008 | + |
| | Inositol 5 | | | |

firmed for sucrose with the house fly in our investigations (table 7). On the basis of these experiments one can assume that a test sugar exhibiting the property of attractance for flies will, when added to 5% sucrose solution, be consumed in greater quantity than 5% sucrose alone. This additive effect has been demonstrated by von Frisch ('34) for all sugars accepted by the bee. If the postulate of additiveness is accepted, then one can also assume that if a sugar cannot

be detected by taste the fly will drink the mixture or sucrose alone in equal quantities. A lower consumption of the mixture than of sucrose alone would indicate a repellent effect of the test substance. Experiments were carried out by offering simultaneously to house flies solutions containing 5% of a carbohydrate plus 5% sucrose or 5% sucrose alone. Table 7 presents the results of these tests. All the compounds which had previously been found toxic had a strongly repelling effect on the fly. Some of them, like xylose and sorbose remained almost completely untouched. Dulcitol was the only non-nutritive sugar which did not diminish the attractiveness of a sucrose solution. This indicates that dulcitol is completely inert to the housefly, a conclusion already arrived at above (page 15) in consumption measurements. These results led us to suspect that repellency and toxicity may easily be confused. Quantitative measurements of the food ingested by an insect are the only good criterion for differentiating between these two possibilities. In the case of the house fly we have seen that both effects exist but as far as our work on the mosquito is concerned, it is impossible to distinguish between the two. This insect consumes such small amounts of solutions that our techniques do not permit sufficiently accurate measurements of consumption to separate the possibilities. *Sarcophaga* has been tested quantitatively with only one carbohydrate. The results were as follows: 5% sucrose alone .108 ml/fly/24 hours. 5% sucrose plus arabinose .076 ml/fly/24 hours. From these figures it may be concluded that arabinose is somewhat repellent but not enough to cause death through starvation, and some toxic effect is therefore apparent.

The above information sheds new light on the results obtained by workers in other laboratories. Hassett ('48) found that *Drosophila* lived for a shorter time when fed arabinose or sorbose in combination with sucrose than they did on sucrose alone. His interpretation of this phenomenon involved the toxicity of arabinose and sorbose. An alternative explana-

tion based on our new information is as follows: These compounds were fed to the insects in M/40 sucrose solution which in itself has a very low nutritional value. If the flies drank less of a mixture containing a particular sugar they would die of malnutrition rather than of a toxic effect of that sugar.

The inhibitory effect of several carbohydrates on the growth of *Tenebrio molitor* should also be discussed in the light of our findings. One of us (Fraenkel, '55) showed that less than 10% of either dulcitol, α -methylglucoside or several pentoses when added to an otherwise adequate diet exerted a pronounced growth inhibition. Subsequently it was observed (unpublished) that *Tenebrio* larvae eat much less of a diet containing 80% of any of the carbohydrates mentioned above than one containing 80% glucose or an inert material like glass or cellulose. The question of whether this is a toxic or repellent effect is not answered, however, and cannot be answered until quantitative measurements of the consumption are made.

Studies on labellar rejection thresholds

In the course of our investigations on the repellency of various carbohydrates for the house fly, it was noted that some of the results contrasted with those obtained by Hassett, Dethier and Gans ('50) with *Phormia regina*. In our studies the house fly rejected xylose and arabinose which were accepted by *Phormia*, as measured by the tarsal reflex. These diverse findings became less difficult to reconcile when it was realized that our tests involved taste receptors situated at the mouthparts, since our method of feeding made tarsal stimulation virtually impossible. The question then arose as to whether substances which are accepted by the tarsi might be rejected by the labella.

Tests were carried out on *Phormia regina* employing the same technique and number of flies used heretofore for repellency studies. It was found that sorbose was neither

sweet nor repellent to *Phormia*, contrary to *M. domestica* which was repelled by it. Both arabinose and xylose, however, were repellent for *Phormia* and at concentrations much lower than tarsal threshold of acceptance as measured by Hassett, Dethier and Gans (table 8). It has, therefore, been proved that a substance may be accepted by the tarsi and yet be rejected by the mouthparts.

TABLE 8

Feeding preferences of Phormia regina

Solutions I and II presented simultaneously in identical pipettes

| TARSAL THRESHOLD * OF ACCEPTANCE (in molar con- centrations) | MOLAR CONCENTRATION OF SOLUTIONS | | QUANTITY CONSUMED OF SOLUTIONS ml/fly/24 hrs. | | SIGNIFI- CANCE OF DIFFER- ENCE P = 1 % |
|--|-------------------------------------|------------------|--|------|--|
| | I | II | I | II | |
| l-Sorbose .140 | Sucrose .143 | Sucrose .143 | .025 | .023 | — |
| | | l-Sorbose .277 | | | |
| d-Xylose .337 | Sucrose .143 | Sucrose .143 | .039 | .016 | + |
| | | d-Xylose .033 | | | |
| | Sucrose .143 | Sucrose .143 | .023 | .008 | + |
| | | d-Xylose .066 | | | |
| l-Arabinose .536 | Sucrose .143 | Sucrose .143 | .069 | .008 | + |
| | | d-Xylose .333 | | | |
| | Sucrose .143 | Sucrose .143 | .043 | .018 | + |
| | | l-Arabinose .033 | | | |
| | Sucrose .143 | Sucrose .143 | .019 | .008 | + |
| | | l-Arabinose .066 | | | |
| | Sucrose .143 | Sucrose .143 | .058 | .008 | + |
| | | l-Arabinose .333 | | | |

* Hassett et al. ('50).

SUMMARY

1. The utilization of 28 carbohydrates by the adults of the mosquito *Aedes aegypti* and the flies *Sarcophaga bullata* and *Musca domestica* was investigated. The insects survived for long periods on glucose, fructose, sucrose, maltose, raffinose, melezitose and sorbitol, and failed to survive on xylose, arabinose, ribose, rhamnose, sorbose, cellobiose, inulin, α -methylmannoside, dulcitol and inositol. There were great differ-

ences between insect species in the survival value of mannose, galactose, trehalose, melibiose, lactose, dextrin, starch, glycogen, α -methylglucoside and mannitol.

2. The presence of the carbohydrases necessary for hydrolysis of all the utilized oligo- and polysaccharides and glycosides, with the exception of melibiase and lactase, was demonstrated in the adult insects. Larvae of *Sarcophaga* and houseflies were shown to possess only invertase, and to acquire the other carbohydrases during the pupal stage.

3. Attempts were made to distinguish between inert, toxic and repellent substances by offering mixtures of non-nutritive substances with sucrose and measuring the food intake of such mixtures. Xylose, arabinose, ribose, rhamnose, sorbose, α -methylmannoside and inositol were found to have a strong repellent effect on the housefly, and in addition to be also toxic. Some of these compounds were found to be toxic and/or repellent also for *Aedes aegypti* and *Sarcophaga bullata*.

4. Xylose and arabinose were found to be rejected by the mouthparts of *Phormia regina* at concentrations even lower than those accepted by the tarsi.

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INTRACELLULAR POTENTIALS IN PACEMAKER AND INTEGRATIVE NEURONS OF THE LOBSTER CARDIAC GANGLION¹

SUSUMU HAGIWARA² AND THEODORE HOLMES BULLOCK

Department of Zoology, University of California, Los Angeles

EIGHT FIGURES

INTRODUCTION

Since Welsh and Maynard ('51) introduced the lobster cardiac ganglion preparation into physiology it has become clear that unique material is here offered for the analysis of the elementary processes of integration and formulation of patterned discharge in nervous centers.

Maynard ('53a, b, c, '55, '56a, b) has shown, besides their neurogenic initiation of the heart beat and their final motor innervation of the myocardium, many of the properties of the 9 constituent neurons (Alexandrowicz, '32) and their probable interactions, as well as the effects of extrinsic inhibitory and acceleratory nerve fibers from the central nervous system. Matsui ('55) has demonstrated many of the same phenomena and offered somewhat different interpretations. Bullock, Cohen and Maynard ('54) briefly referred to the discovery of stretch — or inflation — sensitivity, presumably in the same neurons.

All these studies have been done with external recording electrodes. The present communication is the result of efforts to visualize the electrical signs of activity, including sub-

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² On leave from the Department of Physiology, Tokyo Medical and Dental University, Tokyo, Japan.

threshold events in individual neurons of the several types by the intracellular electrode. A preliminary report has appeared (Hagiwara and Bullock, '55; see also Bullock, '57).

The special features of this preparation are the accessibility and small number of the neurons combined with their integrative functions. Unlike most peripheral ganglia, this one is largely autonomous and not only responds to conditions influencing the heart beat but spontaneously and regularly organizes a complex discharge which has recurrent features permitting the recognition of pattern. The cells are not all alike. There is at the least a division into 5 large and 4 small cells and Maynard has adduced evidence that the latter normally include the pacemaker, the former follow and are the more important as motoneurons. The connections are poorly known, the neuropile appears complex in methylene blue stains and synapses may be upon the cell body or the dendrites or both. Nevertheless it has proven possible to illuminate the virtually isolated ganglion so as to visualize each of the cell bodies and to penetrate most of them at will.

MATERIALS AND METHODS

In most of the experiments California spiny lobsters, *Panulirus interruptus*, were used, but in a few cases the observations were made on *Panulirus inflatus* from Lower California. Specimens varied from 400–2000 gm.

The preparation is very similar to one of those used by Maynard ('55). The dorsal half of the cephalo-thorax is removed. The heart is exposed by cleaning away the deep muscles and pericardium. The carapace is carefully separated from the hypodermis and the latter is also partially removed on the dorsal side of the heart. The cardiac ganglion lies on the inner surface of the dorsal heart wall. The ventral wall is removed under the binocular microscope and that part of the dorsal myocardium immediately around the ganglion leaving the ganglion intact and suspended from the remaining myocardium by its nerves. Some of the smaller nerves are incidentally cut and for internal recording some-

times the remaining larger nerves to the heart muscle are crushed distally because movement frequently prevents the microelectrode tip from staying inside the cell. The preparation is pinned through the remaining heart muscle or hypodermis on a small tilted platform. The central part of the platform is made of transparent plastic through which the ganglion is illuminated by transmitted light. The tilt of the platform brings the long axis of the ganglion nearly parallel with that of the microelectrode. Good illumination makes it possible to distinguish every nerve cell in the unstained ganglion, once some familiarity with landmarks is gained by the use of methylene blue. The best results are obtained with an efficient dark field illumination.

For the internal recording, capillary microelectrodes with an external tip diameter of less than $1\text{ }\mu$, filled with 3 M KCl were used, leading to a DC amplifier with an input cathode follower of low grid current and reduced grid-to-earth capacity (Hagiwara and Watanabe, '56). For the penetration a microelectrode is brought to the ganglion by means of a Peterfi-type micromanipulator, keeping the long axis of the ganglion and that of the microelectrode almost parallel. The outer surface is very tough but weak points are usually found near the branching of the ganglion.

For the external recording, pairs of fine silver wire electrodes made contact with the surface of the ganglion and the potential change was amplified by means of R-C amplifiers with a differential input.

The physiological saline used had the following composition:

NaCl, 26.4 gm; KCl, 1.12 gm; CaCl_2 , 2.78 gm; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.99 gm; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.82 gm; H_3BO_3 , 0.55 gm; NaOH, 0.02 gm; pH 7.1-7.5 (Cole, '41).

Mineral oil was floated on the saline so that the part of the ganglion in contact with the external recording electrodes was in the oil but the region for internal recording was immersed in the saline. All the experiments were carried out at a temperature of $22\text{--}28^\circ\text{C}$.

RESULTS

Through surface electrodes a short burst is usually recorded as shown in figure 1 A, and described by Maynard ('55) and Matsui ('55). The burst repeats with a frequency of about one per second which corresponds to the frequency of the normal heart beat. Complete isolation of the ganglion does not generally change the pattern of discharge. These facts tell us that the cells in the ganglion can

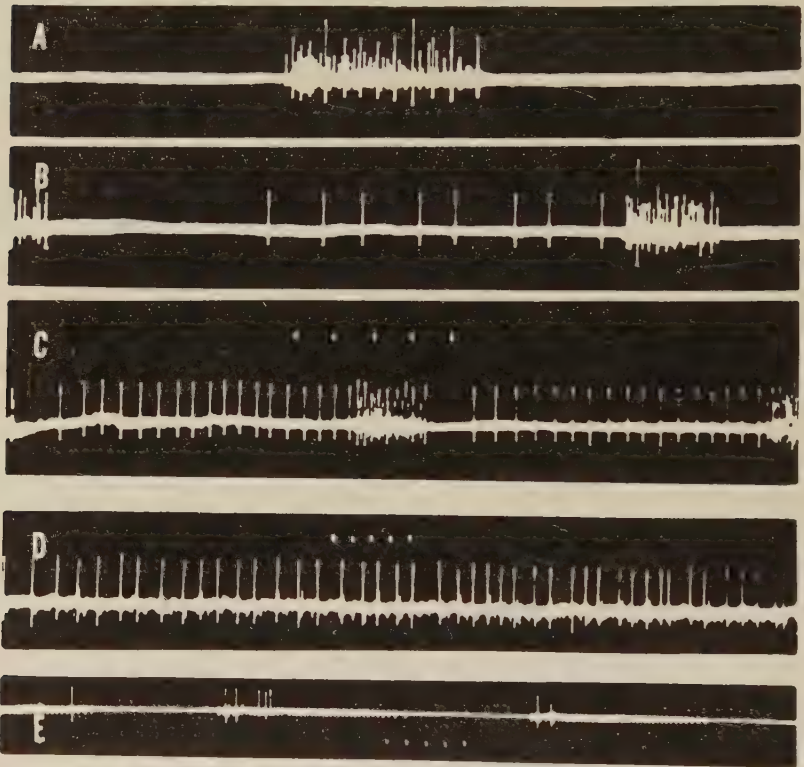


Fig. 1 Discharges from the whole ganglion recorded with surface electrodes. A: Normal burst discharge. In B and C each burst is preceded by a series of impulses of a certain unit. D: Tonic discharge obtained after crushing the small cell pool. E: Burst discharge recorded after reduction of the number of active units. The small amplitude spike is probably from one of the small cells, and seems to set up the spikes of large amplitude trans-synaptically. Time marks 10 per sec. A, B and C are at the same speed.

fire without extrinsic nervous influences and that the discharges of the cells inside the ganglion are grouped. Each burst of the ganglion is composed of spikes of several different neurons and for the most part several spikes from each neuron. Some of these spikes have originated in the neurons which are carrying them. The others presumably have been driven by the spikes of antecedent neurons, hereafter called presynaptic elements. Figure 1 E shows burst discharges of the ganglion recorded after reducing the number of active neurons, by crushing. In this record, as in those analyzed by Maynard ('55) spikes of a smaller amplitude seem to be the spontaneously initiated ones and the larger spikes to be driven by the former. However, it is often difficult to draw such a conclusion from results obtained by surface recording. Simultaneous intracellular recording is helpful in analyzing the sequence of events.

When a microelectrode tip penetrates the surface membrane of a large cell, a potential drop of about 50 mv appears, the inside of the cell being negative to the surrounding solution. Smaller resting potentials are usually recorded from the smaller cells. This does not necessarily mean that the smaller cells have smaller resting potentials. The lower magnitude may be due to damage at the cell membrane by the penetration of the electrode tip and this seems likely to be more serious when the cell is smaller.

The intracellular potential changes of the neurons during the burst can be classified into three different types according to the pattern. These we will call the follower type, the pacemaker type, and the follower type with spontaneity.

Follower type

This type of discharge is the most common one to occur during the normal burst in the large cells and in the medium-sized cells (a category we distinguish within the 4 small cells of other authors). The intracellular potential change of this type characteristically begins with a sudden depolarization

of about 20 mv which is followed by a series of small deflections (2–5 mv) of a relatively slow time course as shown in figure 2, A and B. From the peaks of some of these a spike appears. The amplitude of the spike recorded from inside the nerve cell is very often less than 15 mv and is sometimes as small as 5 mv even when the amplitudes of the slow components of the potential are not different from the other cases.

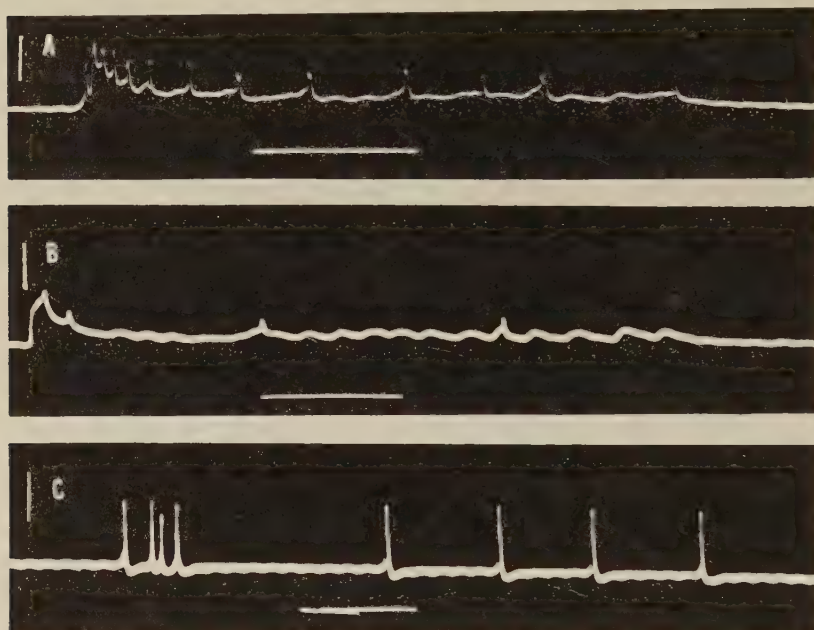


Fig. 2 Intracellular potential change during spontaneous bursts. A and B: recordings from inside large cells. Resting potential is about 50 mv in both cases. C: recording from inside an axon. Resting potential is 56 mv. Voltage calibration, 15 mv. in A and B, 30 mv. in C. Time: 100 msec.

In contrast a spike is occasionally seen which shows a much higher amplitude without the small slow component (C of the same figure). Since no cell body was near the electrode, we believe these cases to represent penetration of axons. The facts suggest that in this ganglion the recorded spike in the cell body is not a real action potential of the cell body itself but a potential which spreads electrotonically from the axon.

The lack of action potential of the cell body does not seem to be due to injury by the penetration of the electrode tip, because the pattern of impulse discharge, recorded from the surface of the ganglion, does not show any noticeable change before and after penetration and the amplitudes of resting and small, slow potentials as well as the pattern persist without deterioration for many minutes or even a few hours. Furthermore, as described below, the normal discharge of this neuron is initiated by the synaptic potential set up in the cell body.

It is not safe to conclude that the cell bodies of the present ganglion are incapable of a proper action potential. In one experiment the recorded spike showed an amplitude of about 30 mv while the slow components of the potential had their usual sizes. The fact that the size of the recorded spike varies in the range between 5 and 30 mv from penetration to penetration in contrast with fairly constant amplitudes of the slow components of the potential suggests that the spike starts at a point distant from the cell and the distance is different from case to case. This naturally leads to the further suggestion that the spike may under some condition start from the cell body itself as an extreme case. However it is clear in any case that the cell can control the initiation of spikes without producing any proper action potential of itself.

When a simultaneous surface recording is made, it is typically found that each small slow deflection in the intracellular record is preceded by an impulse of another neuron. In the case shown in figure 3 B the discharge is tonic instead of in bursts. The surface recording is made between the small cell group and the penetrated neuron; an impulse of small amplitude precedes each small, slow deflection. These impulses are probably initiated from one of the small cells behaving as pacemaker. Some of the small, slow deflections grow up to a spike but others end abortively. From these recordings it must be concluded that there is no certain, critical depolarization above which a spike appears. Sometimes the spike appears in the falling phase of the slow deflection. This again

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indicates that the spike arises at a point some distance away from the cell body. The spike of the penetrated neuron is not to be found in this surface recording possibly because the present cell sends its axon away from the end of the ganglion trunk with the surface electrodes. Figure 3 A gives a similar record when the discharge is in a transition from the tonic type to the burst type. These results lead us to conclude that

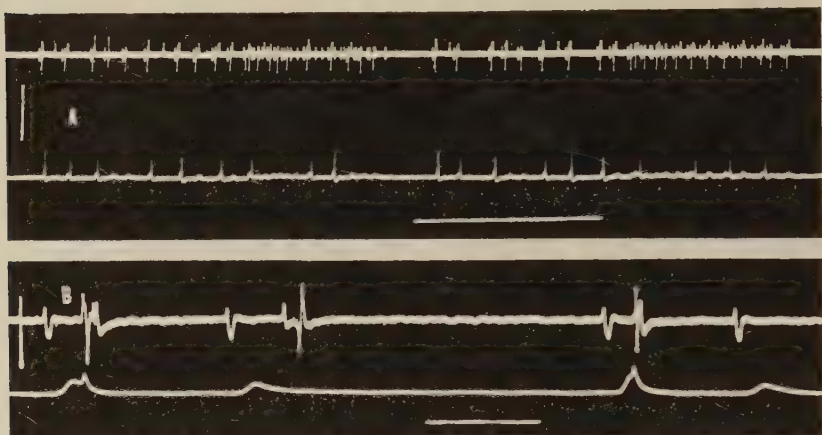


Fig. 3 Simultaneous recording of intracellular potential change of a large cell (below) and surface potential of the ganglionic trunk (above). The surface recording is made between the region of the penetrated cell and the small cell pool. In A the discharge is in a transition from the tonic to the burst type. The variable size of the spikes is unusual; according to the theory in the text it could mean a shifting locus of spike initiation. In B the time scale is expanded. In both cases the intracellular potential change is small, possibly due to injury. Note some synaptic potentials result in spikes, many do not. Voltage calibration for intra-cellular recordings: 10 mv. Time: 1 sec. for A and 30 msec. for B.

the potential which we have been calling a small slow deflection is a synaptic potential and is due therefore to antecedent activity in another unit.

If they are synaptic potentials, similar potentials should be set up by stimulation of the presynaptic fiber. A series of records shown in figure 4 A was taken when the ganglion was stimulated with a single pulse of a short duration, evidently reaching a presynaptic element for the cell penetrated. In

figure 4 A1 the stimulus intensity was weak and it did not cause any potential change of the cell body. With gradual increase of the stimulus intensity, an antidromic spike of the penetrated neuron appeared as shown in figure 4 A2, and

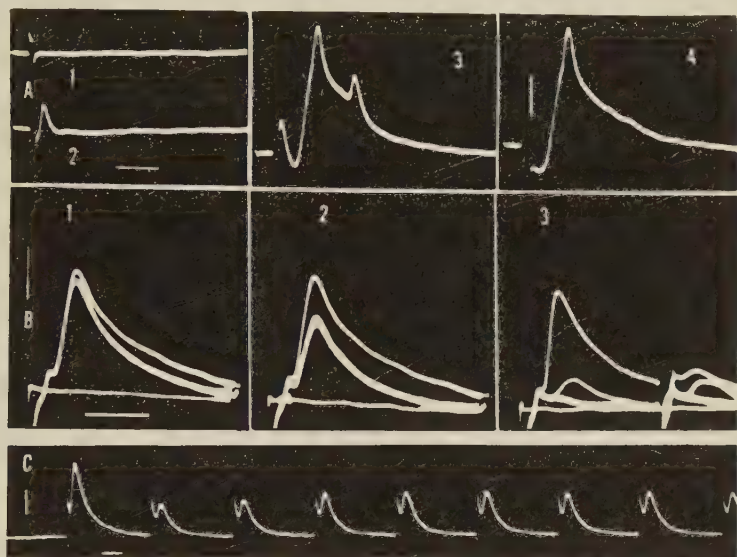


Fig. 4 Synaptic potential recorded from inside a large cell during stimulation of the ganglionic trunk between the penetrated cell and the small cell pool, with a short pulse. A: In 1 the intensity is subthreshold for all. In 2 it is above threshold for the antidromic spike. In 3 the intensity is just above the threshold for the synaptic potential. The synaptic potential in 4 is obtained with a different polarity of the stimulating current. Two and one orthodromic spikes are seen in 3 and 4 respectively. B: The effect of stimulus frequency. The stimulus intensity is supramaximal. Successive responses are recorded on the same film by multiple exposure. The frequency of stimulus is 2/sec. in 1, 12/sec. in 2, and 21/sec. in 3. C: A similar record taken on a running film to show the time course of the effect. Voltage calibration: 10 mv., time: 20 msec.

at a still higher intensity the synaptic potential appeared as shown in figures 4 A3 and 4 A4.

The synaptic potential rises to a peak of about 20 mv and declines exponentially with a time constant of about 25 msec. On the top as well as on the declining phase spike potentials may be superimposed. The number of spikes generated by

a synaptic potential can reach 4. A similar amplitude and a similar time course are found in the initial large deflection observed during the burst discharge.

The amplitude of the synaptic potential behaves almost in an all-or-none manner with change of the presynaptic stimulus. But in some cases a synaptic potential of a smaller amplitude is observed when the intensity of the stimulus is increased very gradually. This means that at least some of the large cells seem to be innervated by more than one presynaptic fiber. Even if the number of the presynaptic fibers innervating a large cell is not one, it can not be many. This is supported by the usual observation that there is a synaptic potential change in intracellular recordings corresponding to only one of the units found in the simultaneous surface recording.

It is known (Maynard, '53a, '55, '56a) that the cardiac ganglion receives three pairs of extrinsic nerve fibers, one inhibitor and two accelerators. These fibers, particularly accelerators, might contribute to the observed small gradations of the size of the synaptic potential. Nevertheless we conclude that a single presynaptic fiber sets up a large synaptic potential and those set up by the others, if they exist, are much smaller in amplitude. The effective presynaptic fiber is very likely to arise from one of the small cells, for the synaptic potential is associated with the unit giving the smallest response in the surface recording. Furthermore it is abolished by crushing the small cells.

Though the amplitude is almost independent of the stimulus intensity, it very much depends upon the stimulus frequency. When a presynaptic stimulus of supramaximal intensity is applied repetitively, the amplitude of the response to the second stimulus is reduced. A slight recovery of amplitude is usually observed in the following few responses and thereafter the amplitude reaches a final level which corresponds to the stimulus frequency as shown in figure 4 C. The recovery observed during the initial few responses is very small so that the steady state amplitude is practically

the same as that of the second response. In figure 4 B a series of responses is photographed on the same film by multiple exposure. In figure 4 B1 the frequency is two per second and the difference between the response to the first stimulus and those to the following stimuli is small. Increasing the frequency causes a larger decrease as shown in the next two figures (4 B2 and 4 B3). Figure 5 shows the relation between the relative amplitude of the steady state synaptic potential and the stimulus frequency. When the frequency

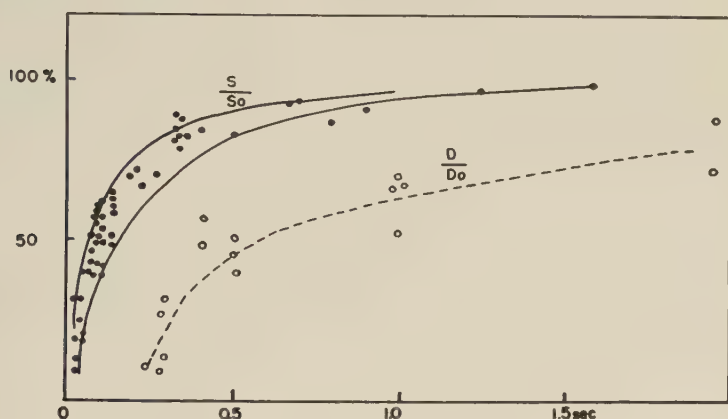


Fig. 5 The effect of stimulus interval (abscissa) upon the amplitude of the synaptic potential (S) and the duration of the reflex discharge of the entire ganglion (D). In both cases unconditioned value S_0 or D_0 is taken as 100. The curves were taken from different preparations.

is changed during stimulation, the amplitude of the synaptic potential shows a change corresponding to the new frequency of stimulus. In this case the ratio between the amplitude of any response and that of the initial response is the same function of interval just preceding as that shown in figure 5. The relative amplitude of the small, slow deflections observed during the burst discharge is also similarly related to the preceding interval. From this evidence the initial larger and following smaller synaptic potentials during the normal burst can be understood as an effect of interval difference: the first

deflection comes after a second or so of quiescence and is quite large, the succeeding potentials come quickly, and are correspondingly smaller. Characteristically the normal burst shows a declining frequency of synaptic potentials and a rising amplitude toward the latter part of the burst.

The above results indicate that a presynaptic impulse causes some effect which lasts much longer than the synaptic potential. There is a possibility that this effect is not due to the presynaptic impulse in question but to the effect of simultaneously elicited impulses in the extrinsic nerves. However, the ratio of the height of any potential to the height of the first potential is the same function of the preceding interval in both the electrically stimulated and naturally occurring cases. It seems quite unlikely that this frequency effect is due to the extrinsic nerves which are silent in the isolated preparation.

Besides the reduction of the amplitude, some reduction of time constant of the falling phase of the synaptic potential is observed during the repetitive stimulation, but no definite relation is found between the two effects. Even a few stimuli applied to the ganglion, too weak to elicit a presynaptic spike, can reduce the time constant from 27 msec. to 15 msec. with little or no effect on the height of the potential. These results indicate that the reduction of the time constant does not seem to be the exclusive effect of the presynaptic spike in question and this conclusion implies that the reduction of amplitude of the synaptic potential is not due to the reduced membrane resistance at the soma.

As described before a single synaptic potential can set up more than one postsynaptic spike. If this is so, a burst-type discharge is expected in the surface recording when a single shock is applied to the ganglion. This is the case, as shown in figure 6. This stimulus frequency is 2 per second in A and 5 per second in B. The relation between the duration of burst and the frequency of stimulation is similar to that between the amplitude of the synaptic potential and the frequency. This is shown in figure 5.

Though it is very much dependent on the stimulus frequency, the duration of the burst does not show measurable gradation with variations of the stimulus intensity. This suggests that only a few fibers can behave as presynaptic elements in the present sense even in the intact ganglion. These seem to be axons from the small cells.

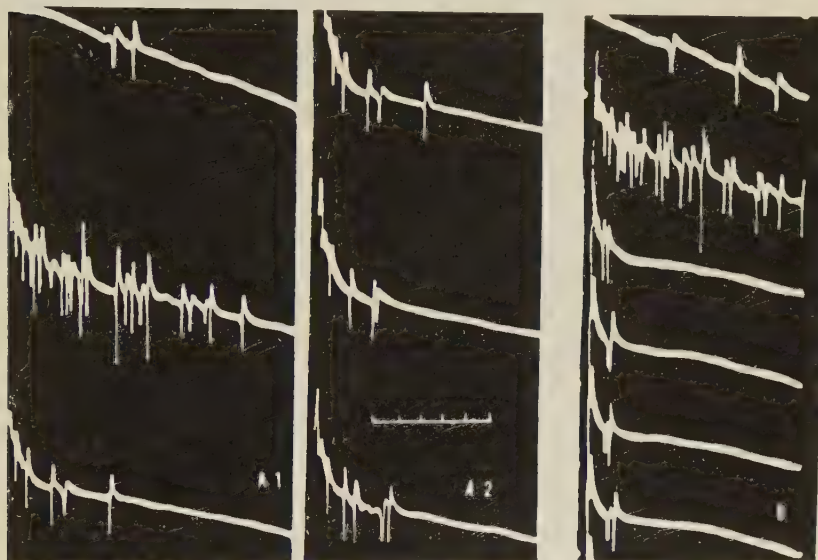


Fig. 6 The effect of stimulus interval upon the duration of the reflex discharge recorded from the entire ganglion. Stimulus frequency is 2/sec. in A, and 5/sec. in B. A2 is a continuation of A1. The lower trace of A1 and the upper trace of A2 are the same. The long burst in each case is an unconditioned response. The following are conditioned responses. Time: 100/sec.

From the foregoing results we conclude that the large and medium-sized cells usually behave as followers during the burst discharge and the small cells seem to be pacemakers. Even if the pacemaker should send only a single impulse per burst, it could still set up a burst discharge of the ganglion, through the repetitive firing of postsynaptic elements and possibly also by cascading of excitation, though this has not been demonstrated.

Follower type with spontaneity

Sometimes the surface recording shows that a normal burst discharge (cf. fig. 1A) is preceded by a series of regular spikes of a certain amplitude as shown in figures 1B and 1C. When the burst discharge is stopped by crushing the caudal part of the ganglion where the small and medium-sized cells are located, the spikes occur at regular intervals without interruption, as shown in D of the same figure.

The intracellular potential change of such a neuron has a characteristic pattern and it will be called the follower type with spontaneity. The potential change during the burst of the whole ganglion is not different from that of the follower type. However, each of the spikes preceding the burst is preceded by a gradual depolarization instead of a synaptic potential as shown in figure 7A. These spikes we believe to originate in the penetrated neurons for the following two reasons. There is no noticeable potential corresponding to a presynaptic spike found in any recording through surface electrodes. Also, an exactly similar potential change, often called the generator potential, has been observed by a number of authors at sensory endings (Eyzaguirre and Kuffler, '55a,b), at the pacemaker of the heart (vide Weidmann, '51; Trautwein and Hutter, '56) and at the locus of direct current stimulation (Hagiwara and Oomura, '56). In the experiment shown in figure 7B the cell is firing spontaneously with a tonic discharge of about 10 per second. An antidromic stimulus applied to this neuron resets the rhythm of firing while presynaptic stimulation sets up a synaptic potential which causes a number of spikes. The synaptic potential is always followed by a period of repolarization during which there is no spontaneous discharge. Thereafter, gradual depolarization begins and spikes begin to fire with a lower frequency than before and the rhythm usually recovers to the original level within the first few spikes. This means that such series of spikes of a given amplitude, just preceding a burst, are not a part of nor

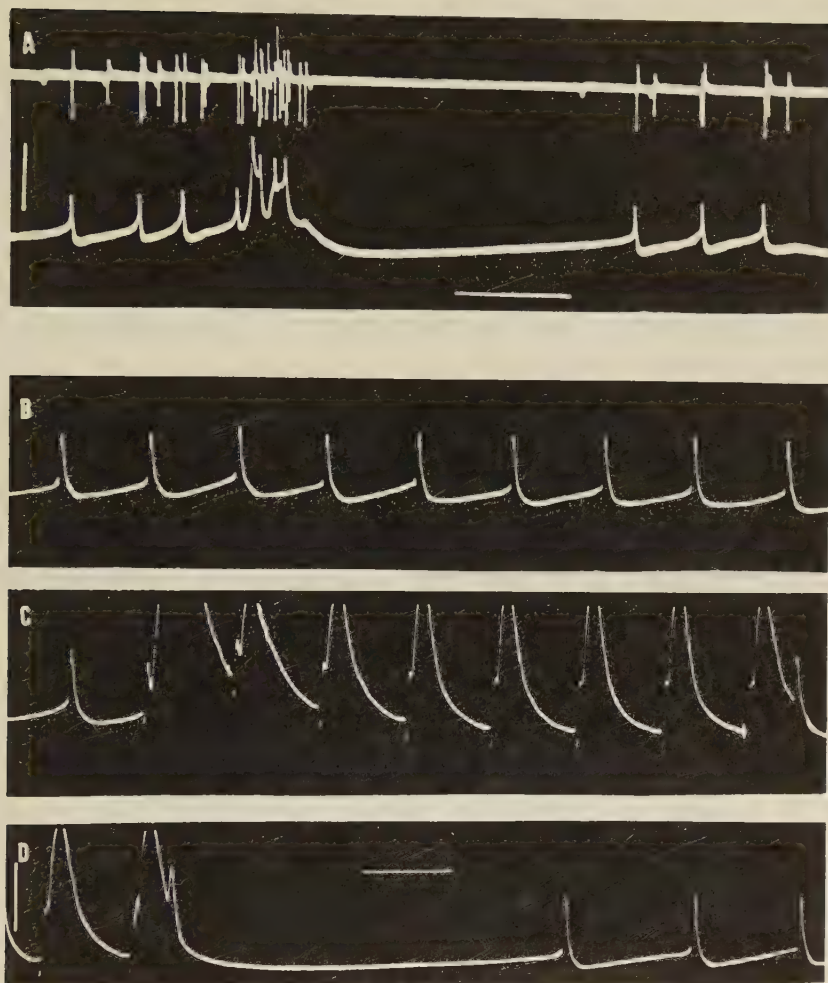


Fig. 7 Intracellular potential changes of the large cells. A: Follower type discharge with spontaneity. Upper trace shows the simultaneously recorded impulse discharge of the ganglionic trunk. Voltage calibrations for the intracellular potential: 10 mv., time: 100 msec. B: The penetrated large cell shows a spontaneous discharge of the tonic type. A gradual depolarization leads to a spike without any synaptic potential. C: A series of stimuli applied to a presynaptic neuron causes synaptic potentials and occasionally orthodromic spikes. D: A continuation of C. A silent period is followed with a spontaneous discharge of a reduced frequency. Voltage calibration for B, C and D: 10 mv., time for B, C and D: 100 msec.

anticipating that burst but are really an escape of a non-pacemaker cell with sufficient spontaneity from the depression following the last burst.

From these results we conclude that the burst phase of all the cells is synchronized, that is, the cells all fire during the same short period, though not necessarily at exactly the same time. This is true as long as a pacemaker neuron continues to fire and without too short an interval since the last burst, even when some follower cells have independent spontaneous discharges.

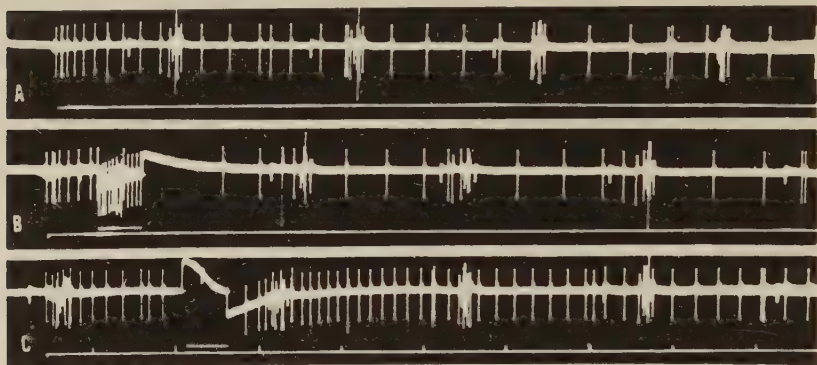


Fig. 8 Stimulation of a large cell through an internal electrode. The excitation of the neuron is observed by recording the impulse of the neuron through surface electrodes. A: Impulse stimulation with a long lasting outward current. B: A short outward pulse of a stronger intensity is superimposed on the same current. C: The superimposed current is inward. Time: 10/sec.

The synaptic potential has some depressing effect on spontaneous firing. This depression does not seem to be specific to the synaptic potential but to any long and large depolarization. In a few experiments the cell body was stimulated through an internal electrode with a direct current and spikes of the same neuron were recorded extracellularly as an indication of excitation. A current intensity of 10^{-8} amps. was sufficient to cause a repetitive firing, when it flowed through the membrane in the outward direction.

When a strong current pulse in the outward direction is superimposed on the direct current, the frequency of discharge is greatly increased during the pulse and this is followed by a period of depression. A pulse of the same intensity and the opposite direction caused an opposite effect (fig. 8).

Pacemaker type

Sometimes one cell, especially one of the small cells, shows a repetitive burst of impulses with constant impulse intervals after most of the other cells have become silent (cf. fig. 1E). No successful intracellular recording was obtained for this pattern from a small cell. A large cell, which has been spontaneously discharging with a tonic discharge, sometimes begins to show similar repetitive grouped discharges and this change is reversible. The corresponding intracellular record, which is often obtained, is not much different from that of the tonic one. The difference is only that the process of gradual depolarization recurs equally after each spike in the case of the tonic discharge while in the case of the bursts it is intermittent.

DISCUSSION

The mechanism of the spontaneous discharge of the cardiac ganglion has been investigated by Maynard and by Matsui with external surface recording. The present experiments show good agreement with these results. Maynard explained the spontaneous burst discharge of the ganglion by assuming extensive nervous connections among the cells and an essential interaction in the form at least of positive feedback. In the light of the new results we can say that the properties of pacemaker action upon followers are certainly crucial in determining the pattern of the burst and that cascading is possible, though not directly supported, but as far as the evidence goes it does not confirm — or exclude — the existence of reciprocal action or feedback and hence of extensive inter-connections among the cells.

We can confirm Maynard's conclusion that in the normal ganglion the small cells behave as pacemakers and the large (and medium-sized cells) are fired by impulses from the pacemakers and that the correspondence between the presynaptic and postsynaptic spikes is not 1 : 1 and not necessarily constant though maintained for some time in the normal beating heart. The number of postsynaptic spikes will be larger or smaller than that of the presynaptic spikes according to whether the number as well as the frequency of the pacemaker spikes is small or large.

The pacemaker can control the follower even though the latter discharges spontaneously. The variable relation between the numbers of presynaptic and postsynaptic spikes, and this property of subservience of the follower cell operate in an automatically regulatory fashion tending to maintain the burst of the whole ganglion approximately constant even when some change occurs in the pacemaker or follower.

It may be worth while to discuss the multiple spikes caused by a single synaptic potential. This may be explained in terms of the conditions which prevent the spike from entering the cell body where the synaptic potential originates or at least is large. According to Coombs, Eccles and Fatt ('55) the synaptic potential is caused by an instantaneous short circuit of the resting membrane and the falling phase of the potential is a simple discharge of the charged membrane capacity through the resting membrane resistance. If this were so in our preparation, a spike in the cell body would return the membrane potential to the resting level. Furthermore, and as a consequence, multiple firing would never be expected if the falling phase occurs during the falling phase of the synaptic potential. However, if the spike starts some distance away, in the axon, and the cell body does not show a proper action potential, the acceleration of the falling phase of the synaptic potential during the falling phase of the spike will occur mainly only in the nearby axon. The observed electrotonic potential in the cell body attributable to the spike is small (5-10 mv) compared with the spike observed

presumably in the axon (60–70 mv). If the synaptic potential were reduced by a similar amount in spreading from the soma to the region of spike origin, it would not be sufficient to set up a spike. However, the effective space constant is much larger for the slowly changing potential such as the falling phase of the synaptic potential. Therefore, the slow fall of the potential of the soma permits its electrotonic effect to be felt strongly at some distance in the axon whereas the rapid course of the spike in the axon prevents its effect from being felt strongly in the cell.

We should comment on the lack of a proper action potential in the cell body in relation to other reports. In other preparations such as the cell body of the crustacean muscle-receptor organ (Eyzaguirre and Kuffler, '55a, b), the motoneurons of some insects (Hagiwara and Watanabe, '56), and large ganglion cells of gastropods (Arvanitaki and Chalazonitis, '55; Tauc, '55) there seems to be no reason to doubt the presence of a proper action potential. Tauc ('55) speaks of pseudo-spikes which are larger than synaptic potentials but distinct from spikes in the same cell. Fatt ('56) concluded from an external potential field analysis that the action potential of the soma of mammalian motoneurons has a component which is quite different from the action potential of axon or dendrite and the cell can produce this component without setting up an ordinary spike. This potential has a small amplitude (30 mv) and an exponential decay with a time constant regarded as that of the resting membrane and summates with the synaptic potential. Though the spike recorded in our preparation has some properties similar to those of the above, we conclude that the recorded spike in our case is not comparable but is an electrotonic potential at the cell body due to the spike of the axon nearby. But it is still inconclusive whether the present cell can under some conditions produce a proper action potential.

Although contrasting in respect to the properties in the last two paragraphs, there are many parallels between the

present cell types and those in the ganglia of snails and sea slugs (Arvanitaki and Chalazonitis, '55; Tauc, '55). These include the lability of the firing voltage and of synaptic potentials, the importance of slow depolarizations and rebound phenomena. We have not seen truly oscillatory potentials like those of Arvanitaki and Chalazonitis ('55).

SUMMARY

1. Each of the 5 large and two medium-sized cell bodies of the virtually isolated ganglion has been penetrated without abolishing for considerable periods the sustained activity similar to that seen before penetration. Under good conditions this is a patterned burst repeated at heart beat frequency.

2. Resting potentials are generally about 50 mv, in the larger cells, somewhat less in the smaller cells.

3. Changing potentials are classified into the follower and the pacemaker types and the follower with spontaneity.

4. The follower type of potential is most common. Each burst is signalled in such cells by a sudden depolarization of about 20 mv. followed by a series of small (2-5 mv.) slow (30-40 msec.) deflections. From the peaks of some of these small spikes (5-30 mv.) arise.

5. The evidence indicates that the soma normally has no real spike but only small, electrotonic reflections of the axonal spike, which originates some variable distance from the soma. The soma nevertheless controls the initiation of spikes. There is no constant level of depolarization which is associated with the initiation of a spike.

6. The small, slow deflections are concluded to be synaptic potentials resulting from the arrival of a single presynaptic impulse. Each synaptic potential is capable of initiating one to 4 spikes.

7. Some large cells receive only one presynaptic fiber, indicated by an all-or-none effect of presynaptic stimulation. Some cells show a small additional effect of supraliminal

stimulation and hence receive more than one presynaptic fiber but in any case only very few.

8. The presynaptic neurons responsible for driving these large and medium-sized cells are the two small cells.

9. The interval between arriving presynaptic spikes greatly influences the size of the synaptic potential. The effect can be called defacilitation—shorter intervals are followed by smaller response. This is regarded as a part of the normal mechanism of regulation of discharge.

10. Presynaptic impulses or subthreshold direct stimuli reduce the time constant of the synaptic potential but not in parallel with the amplitude.

11. Potentials of the follower type with spontaneity are similar to the above except that between normal bursts spikes occur which are preceded by a gradual depolarization similar to that found in the pacemaker region of other tissues. Presynaptic stimulation elicits a synaptic potential which blocks this and prevents it from restarting for a short time. A pacemaker can control and synchronize a burst discharge even when the followers have their own spontaneity.

12. DC polarization with an intracellular electrode and about 10^{-8} amps. causes repetitive firing with current leaving and suppression of activity with current entering the cell. At the OFF of such currents a strong rebound effect occurs.

13. No penetrations caught a cell which was at that time pacing others in a normal burst pattern. From the cases of spontaneously firing cells it is thought that the pacemaker type of potential is essentially the gradual depolarization leading to a spike, described above, but firing intermittent bursts.

14. The lack of a real spike in the cell body is interpreted as an adaptation associated with the repetitive firing and continued control by the soma during the falling phase of the synaptic potential. The slow soma potential can spread electrotonically into the axon to the spike initiating region with little decrement whereas the spike, being severely attenuated in the reverse direction due to its brevity, does not repolarize the soma and abolish the residual synaptic potential.

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THE ADSORPTION OF LIPIDS FROM THE ERYTHROCYTE SURFACE BY SILICA AND ALUMINA

E. A. BROWN

*Department of Physiology, Medical School, University of Birmingham,
England*

During investigations of the effect of certain mercurated resins on the human erythrocyte surface, it was found that Hyflo-Super-Cel,¹ used as a supporting material in the column, rapidly haemolyzed the cells. This paper reports the results of experiments to determine the nature of this haemolytic effect, and discusses its possible applications to the problem of the surface structure of the erythrocyte.

Outdated bank blood was the chief source of erythrocytes in these experiments, and on one occasion a bottle of blood only three days old, which had been rejected as unsafe to use for transfusion, was used. A suitable sample of the blood was removed, with sterile precautions, and centrifuged at 3,000 r.p.m. to separate the plasma. The cell deposit was resuspended in an equal volume of a M/6 solution of A.R. NaCl in glass-distilled water ("saline"), centrifuged, this operation being repeated three times. Then the cell deposit was transferred to a 500 ml centrifuge pot, and suspended in 500 ml of saline, centrifuged, and the supernatant discarded. The cell deposit was again resuspended in 500 ml of NaCl solution, centrifuged, and the supernatant removed and kept.

The washed cells were now resuspended in 500 ml saline. Into a thoroughly cleaned glass tube 158 cm long and 3 cm bore, closed off at one end except for an outlet tube, was placed 250 ml of a 20% solution of sucrose. On top of this was floated the 500 ml of cell suspension, and on this about

¹ Obtained from Johns-Manville Corp., U.S.A.

100 ml of saline. A measured quantity of Hyflo-Super-Cel was taken — usually 10 g — and suspended in 50 ml of saline. In two experiments activated alumina was used instead. The suspension was poured into the tube containing the cell suspension, and the powder particles allowed to pass down, through the cell suspension, into the sucrose solution at the bottom. The purpose of this arrangement was to bring the cells and the powder particles into contact, and then separate them quickly so as to expedite the washing of the powder. After it had settled to the bottom of the tube the powder was run off into a 500 ml conical flask, and then washed by addition of successive 250 ml amounts of saline, and decanting after the powder had re-settled. The finer particles were rather slow in settling and much of this fraction of the powder was sacrificed for the sake of speed. This step was also indicated by the need to get rid of the red cells in the suspension, since too long an interval between washings would have allowed some sedimentation of these to occur, increasing the length of the whole process. With experience it proved possible to free the powder from red cells without losing more than a small fraction of the powder. After this, washing was continued by putting the powder into a large filter funnel lined with coarse filter paper, and running through about 4 litres of glass-distilled water, with frequent stirring. The total quantity of washing fluid was 8–10 litres.

The supernatant fluid from the final washing of the cells was also mixed with 10 gm of Hyflo-Super-Cel, and the latter subsequently washed by decantation with saline and distilled water, just as described. Finally, the powders were filtered, dried in a hot-air oven at 40°C. for 24 hours, and weighed. The weight so obtained does not pretend to great accuracy.

Each powder was now transferred to a 250 ml flask, and to it was added 100 ml of a mixture of three parts chloroform and 1 part methanol. The chloroform was B.D.H. laboratory reagent grade (non-volatile matter < 2 mg%) and the methanol was double-distilled from B.D.H. technical grade methyl alcohol. The suspensions were stood with frequent shaking

for one hour, and then filtered. The filtrates were reduced to dryness by distilling off the solvent at 40°C. under vacuum, in previously weighed flasks. The residues were dried further in a desiccator, and weighed next day. Each was then extracted with two 5 ml portions of acetone, and the extracts transferred to conical centrifuge tubes. The acetone was driven off by gentle heating over a water bath, and the dry residue redissolved in a mixture of equal parts acetone and ethanol. Any insoluble matter at this stage was removed by centrifugation, and the clear supernatant solution finally evaporated once more. Cholesterol in this material was determined by a modification of the Schoenheimer-Sperry technique (Sperry and Webb, '50).

The remainder of the residues, after this extraction with acetone, was re-dissolved in 10 ml of 3 : 1 chloroform-methanol mixture. Five milliliter of this was removed, and placed in a Kjeldahl digestion flask. The remaining 5 ml was transferred to a boiling tube. The flask was washed out with a further 5 ml of chloroform-methanol, and the washings added to the material in the boiling tube. Nitrogen was determined on the first 5 ml aliquot by a modified micro-Kjeldahl method (Ma and Zuazaga '42). Phosphorus was determined on the second 5 ml aliquot by a modification of the method of E. J. King (King, '39).

In four of the experiments described, 1 ml of the final cholesterol-free extract was taken to dryness, and redissolved in 2 ml of 10% benzene in iso-propyl alcohol. A Langmuir film balance was prepared by cleaning a silica trough (of 3 l capacity) in chromic acid for 4 hours, and washing in running water for 8 hours, then in several changes of de-ionized distilled water. This was then filled with de-ionized distilled water, and the surface swept. After adjusting the movable barrier and torsion-head boom, the presence of foreign matter on the water surface was tested for by moving the barrier up and noting any deflection of the mirror on the torsion head. When the cleanliness of the surface was established, 0.5 ml of the benzene-isopropyl alcohol solution of the extract was

spread on the water surface. This resulted in a large increase in surface pressure, and deflection of the mirror, which was measured by the movement of a reflected light beam. By adjusting the torsion head the reflected beam was restored to its zero position, resulting in increased film pressure. A suspension of Hyflo-Super-Cel in de-ionized distilled water containing 4.5 gm in 10 ml was made, and mixed thoroughly. One milliliter of the suspension was then injected rapidly below the film, the stream being directed upwards towards the underside of the film. Any decrease in the surface pressure was restored by decreasing the area of the film, and this change of area was used as a measure of the effect of the Hyflo-Super-Cel. Besides this, small quantities — about 2 mg — of the powder were blown across the film surface, and changes in surface pressure measured as before. The details of the technique used in these experiments with the film balance are described in a doctoral thesis by P.H. Anderson (Anderson, '54).

RESULTS

Estimates of the three fractions obtained from chloroform-methanol extracts of the various powders after contact with human erythrocytes are given in table 1. Values for cholesterol and phospholipid in similar extracts from powders mixed only with the final 500 ml of washing fluid are included for comparison. In view of the very low values for the first two fractions, it was not deemed worth while to estimate nitrogen in these latter extracts.

The experiments with the film balance showed that there was a large decrease in surface pressure when a suspension of Hyflo-Super-Cel was injected from behind the movable barrier, in such a way that the particles came close to the polar side of the film. This was essential — merely injecting the suspension into the water below the film had no effect. Blowing a very much small quantity of the powder across the non-polar surface of the film caused as great, or even greater, decrease in surface pressure. The changes in the area of the

film produced in this way are shown in table 2. In each case, the area decrease was that required to restore the original surface pressure after the injection or deposition had been made.

TABLE 1

| VOL. OF CELLS TREATED | WEIGHT OF POWDER RECOVERED | TOTAL EXTRACTED MATERIAL | CHOLESTEROL | PHOSPHORUS AS LECITHIN | PROTEIN |
|--------------------------|----------------------------------|--------------------------------|-------------|---------------------------|-----------|
| <i>ml</i> | <i>gm</i> | <i>mg</i> | <i>mg</i> | <i>mg</i> | <i>mg</i> |
| 15.5 | 9.6 ¹ | 2.7 | 0.308 | 0.20 | 0.187 |
| 15.5 ² | 6.0 ¹ | 1.0 | 0.196 | 0.143 | 0.0 |
| 15 | 8.9 | 10.6 | 1.124 | 3.43 | |
| 14.5 ² | 9.1 | 9.5 | 0.428 | 1.44 | 0.536 |
| 16 | 5.7 | 7.2 | | 0.415 | 0.262 |
| 15 | 9.2 | 12.3 | 2.7 | 2.45 | 0.836 |
| Cell-free supernatant | 8.20 | 1.0 | 0 | 0 | |
| Cell-free supernatant | 7.1 | .. | 0 | 0.07 | |
| Cell-free supernatant | 7.4 | 0.9 | Trace | 0 | |
| Cell-free supernatant | 7.5 | 0.7 | 0 | 0.05 | |

¹ Activated alumina used.

² The cells of the preceding experiment were exposed to a second lot of powder.

TABLE 2

| OPERATION | | INITIAL FILM AREA | FINAL AREA | % DECREASE IN AREA |
|------------------------------|---|-------------------------|-----------------------|-----------------------|
| | | <i>cm²</i> | <i>cm²</i> | |
| Injection of 0.45 gm powder | a | 240 | 221 | 8 |
| below water surface | b | 248 | 235.5 | 5 |
| Deposition of c. 2 mg powder | a | 240 | 218 | 9.1 |
| on film surface | b | 218 | 194 | 10.9 |

DISCUSSION

These experiments show that the rapid and extensive haemolysis which is observed whenever a suspension of washed human red cells is brought into contact with particles of Hyflo-Super-Cel is probably due to the adsorption of lipids and possibly lipo-proteins from the cell surface. The powder is completely inert chemically and there is no possibility of

reactions in solution taking place. Two possible modes of action may be envisaged — either the adsorbent removes from solution lipids with which the cell membrane components are in equilibrium, or that contact between cell surface and particles of the powder gives rise to an interaction in which lipids are removed directly from the surface. Since this work was completed, the results of Lovelock ('55) have come to my notice, and they are very relevant to the hypotheses under consideration. He has shown that washed human red cells maintained in contact with alumina haemolyze slightly and slowly and this is in accordance with the results I have obtained. From this and other evidence the conclusion is drawn that solid adsorbents exert their action by removing dissolved lipids, derived from the structural components of the cell. Much of the experimental evidence presented here is, however, not wholly in agreement with that view.

Firstly, there is the fact that haemolysis by Hyflo-Super-Cel is very rapid and affects a large fraction of the cells exposed at once. Now if the hypothesis advanced by Lovelock is applied to this finding, it must mean that Hyflo-Super-Cel is a far better adsorbent for dissolved lipids than activated alumina. The alumina used in my experiments was commercial chromatographic alumina, of particle size about 100–150 μ , washed and heat-treated. It has been tested for adsorption capacity towards phospholipids, and is highly active. For example, purified phosphatidyl serine in chloroform solution is adsorbed and cannot be eluted with the same solvent. In contrast, Hyflo-Super-Cel does not retain this material (Hawthorne and Dils, '56). Generally speaking, Hyflo-Super-Cel does not appear to be comparable with alumina as an adsorbent, for lipids in solution. This certainly applies when the solvent is organic, though of course conditions may be different where aqueous solutions (or dispersions) are concerned. At present there seems to be no available information on this point. It may be argued against this that comparison of Hyflo-Super-Cel and alumina on a weight basis is misleading, and that a given weight of the former

presents a much greater adsorptive surface than the latter. This does not appear to be the case however. Iler ('55) gives 20 m^2 per gram, as the maximum specific area approached by commercial processed diatomaceous earths such as Hyflo-Super-Cel, and Reitz ('49) gives 200 m^2 per gram as the specific surface of activated alumina. The actual figure for any particular sample of activated alumina may vary considerably from this, and depends of course on the treatment which the sample has received. But at the very least it seems that the specific surfaces of the two different materials should be comparable.

Hyflo-Super-Cel and alumina may also be compared with respect to particle size. The particles of the former are certainly of smaller average diameter as judged by sedimentation velocity. They may reasonably be compared to another product derived from the same source Dicalite Speedplus which has between 69 and 94% of its particles in the $2\text{--}20 \mu$ range (Hull et al, '53). If the much greater haemolytic effect of Hyflo-Super-Cel is not due to superior adsorptive capacity for dissolved phospholipids, nor to a greater active surface per gram, then it may be that its smaller average particle size allows greater collision frequency between cells and particles, suggesting an actual removal of material directly from the cell surface. One other possibility is that the amorphous silica of which Hyflo-Super-Cel largely consists has some particular property of surface configuration which enables it to interact with the cell surface in a specific manner.

The evidence of the experiments with the Langmuir film balance suggests that actual contact between the particles and the film is necessary for the removal of material from the film. This is admittedly a rather crude model, but the material of which the film was made had been obtained from red cells by adsorption onto Hyflo-Super-Cel in the first place, and had been handled in very mild conditions. It, at any rate, gave no sign of being adsorbed from solution by Hyflo-Super-Cel, because over periods of 1-2 hours the surface pressure

stayed constant in the presence of several grams of the powder.

It seems likely that at least two modes of haemolysis by solid adsorbents may exist. In the case of Hyflo-Super-Cel the evidence brought forward supports the hypothesis of actual interaction between the cell surface and the particles of the solid, which may or may not be accompanied by adsorption of dissolved lipids, derived from the cell surface. In the case of alumina Lovelock ('55) has postulated this latter process to be solely operative, but the possibility of direct interaction is not ruled out. Further experiments are needed to assess the relative importance of the two processes, and these are being undertaken.

I am indebted to Dr. J. R. Majer of the Chemistry Department, University of Birmingham, for much constructive advice and criticism in the course of this work.

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MEMBRANE RESTING AND ACTION POTENTIALS FROM A PROTOZOAN, *NOCTILUCA* *SCINTILLANS*

MITUHIKO HISADA¹

Misaki Marine Biological Station, Kanagawa-ken, Japan

EIGHT FIGURES

Since the glass capillary microelectrode for the measurement of resting and action potentials of the single muscle fiber was developed by Gerard and his co-workers (Graham and Gerard, '46; Ling and Gerard, '49), the electrical potential differences across the membranes of various cells and tissues have been measured extensively, especially in muscle and nerve of higher animals. However, very little is known about the potentials of protozoan cells. Kamada ('34) detected considerable resting potential across the membrane in *Paramecium*. This potential varied with the nature of the surrounding solution. More recently, in *Opalina*, Kinoshita ('54) has found a clear correlation between the p. d. values across the cell membrane and the patterns of the metachronal wave of ciliary beating.

For the present investigation the luminous flagellate *Noctiluca scintillans* was selected because of its large size and its tentacular activity. Inside-outside resting and action potentials were measured by means of both potentiometric and oscillographic devices.

MATERIAL AND METHODS

Specimens of *Noctiluca* collected from the sea were kept standing in Van't Hoff's artificial sea water for two to three

¹ Present address: Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

hours. (The sea water formula was: 5/9 M NaCl, 10/27 M CaCl_2 , 10/27 M MgCl_2 and 5/9 M MgSO_4 , mixed in a volume ratio of 100: 2.2: 3.0: 11.7: 3.8, respectively. The pH was adjusted to 8.2, using NaHCO_3 .) Then cells having a diameter of about $350\ \mu$ were selected for study.

The cell was brought into a glass chamber constructed so as to permit microscopic observation and, at the same time,

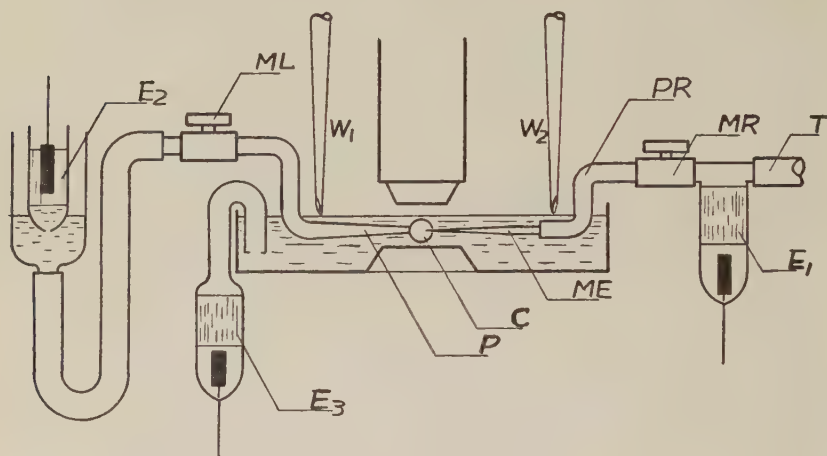


Fig. 1 Diagram of experimental arrangement. ME: capillary microelectrode. C: single cell. P: micropipette to hold the cell in position. E₁: non-polarizable electrode connected to the microelectrode. E₂: indifferent electrode. E₃: spare indifferent electrode. W₁: water inlet. W₂: water outlet. MR, ML: cramps of right and left hand micromanipulators to hold the microelectrode and micropipette. PR: glass probe holding the micropipette and connecting it electrically to E₁. T: plastic tubing connected to an injection syringe.

determination of the electric potential (fig. 1). The specimen, immersed in fluid, was held under the microscope by a sucking device, a micropipette (P) mounted on a left hand micromanipulator (ML). The tip diameter of the micropipette was about $100\ \mu$. Inside back pressure held the cell (C) at this tip, being regulated by the up and down motion of an indifferent electrode vessel (E₂). A capillary microelectrode (ME) was held by a hollow glass probe (PR) which connected it electrically with a non-polarizable electrode (E₁) and, by

means of plastic tubing (T), with a syringe. These elements were mounted on a right hand micromanipulator (MR) so that the microelectrode could be inserted horizontally into the cell. Experimental solutions were made to flow through the chamber at constant velocity. The mechanical agitation caused by this constant flow was studied carefully; no shifting of potential can be attributed to this agitation. The level of the medium was kept constant by means of a water inlet (W_1) and outlet (W_2). When the medium in the chamber was changed, it was also changed in the micropipette. In order to do so, the cell was released from the tip where it had been held. During this period an indifferent electrode (E_3) was placed in the circuit instead of E_2 .

No evidence was found that the illumination used during the microscopic observations had any effect on shifting of the potential.

Resting potentials were obtained potentiometrically by using a capillary electrometer as a null instrument. It was desirable for accuracy that the overall resistance of the input circuit be not too high. Therefore, microelectrodes of fine tip diameter, such as $0.5\ \mu$ or less, and having a resistance of perhaps more than 20 megohms, were avoided. Instead, microelectrodes with rather wide tip diameters, of about $2\ \mu$, were used. These were drawn from hard glass tubing and filled with a solution of 3 M KCl, after Nastuk and Hodgkin ('50), by poking out air bubbles from the tip with a fine glass fiber. In microelectrodes of this diameter there is ordinarily diffusion of KCl from the tip, at first rapidly and then at a steady rate, when the tip is submerged in either an experimental solution or protoplasm. To avoid damage to the cell from contact with the concentrated KCl, it was sufficient to apply slight back pressure to the inside of the electrode. The pressure was regulated by a screw adjustable injection syringe installed at the rear end of the electrode probe. Two criteria facilitated this adjustment, first, portions of solution leaking from the tip were faintly visible in contrast with the outer medium because of the difference between refractive indices

and, second, if the outer medium was sucked in it caused a readily discernible sudden change in refraction at the tip. An outflow of KCl invariably resulted in an instant fall of measured potential differences and in destructive contraction of the protoplasmic strands inside the cell. Measured values which were succeeded by such changes were abandoned. The injection syringe also made it possible to refill and clean the electrode by forcing out KCl solution from the tip and rinsing with distilled water.

The observation of action potentials were made with a cathode ray oscilloscope, the high input resistance of which allowed the use of fine microelectrodes. The microelectrode was connected to a cathode follower tube through a Ag-AgCl type non-polarizable electrode, and the output of this step was fed to a d-c amplifier and read on a cathode ray tube. Measured potentials were calibrated with a low resistance voltage calibrator inserted between the ground and the indifferent electrode.

RESULTS

Resting potential. The potential difference between the inserted electrode and the indifferent electrode is found to undergo a sequence of change in correlation with the demeanor of the protoplasm at the tip of the inserted electrode.

If the tip of a microelectrode with a diameter of about 2μ is brought into contact with the surface of the cell and then gradually pushed forward to penetrate the cell wall, it is observed that the outermost pellicle of the cell shows appreciable rigidity and resists the impalement. Consequently, the electrode is apt to pop in through the thin layer of protoplasm, rarely coming to a stop precisely within this layer. It seems quite probable, therefore, that the electrode greatly damages the pellicle and protoplasmic layer. This might be expected to result in low values of measured potential. As actually observed, there is no potential change immediately after the impalement, then within half a minute there appears a small potential difference which shows no regularity in

magnitude but never exceeds several millivolts negative to the external solution. This is succeeded by a rather rapid increase of electro-negativity at the tip of the internal electrode and the measured potential finally attains a stationary value of 48 ± 2.3 mV, calculated from 28 measurements at 19.0 to 20.5°C . (fig. 2).

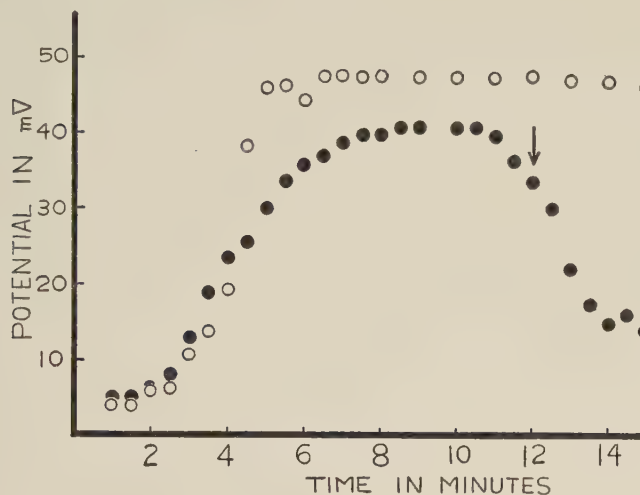


Fig. 2 Two representative changes in potential values measured potentiometrically with microelectrode of 2μ tip diameter. Ordinates indicate the potential negativity relative to the medium outside abscissae the time after impalement. A normal sequence of the change is shown in the hollow circles. Solid circles were plotted with the data of a case in which the vacuole formation around the tip of electrode was discerned at the point indicated with the arrow. Electrode was placed in the cell sap close to the thin layer of protoplasm. Temp. 20.5°C .

Thirteen measurements in which potentials of less than 20 mV were obtained were excluded since they were made during an early stage of the investigation and such low values could not be repeated later except in a few cases which apparently ensued from faulty impalements.

The change in potential values corresponds to the demeanor of the protoplasm around the tip of the electrode: that is, when the adjacent protoplasmic strands are flowing toward the electrode to heal the injury and to make a tight sealing

around it, a small potential is observed, but when a mass of protoplasm finally reaches and encloses the tip of the electrode, the measured potential instantly begins to grow toward the stationary value. This sequence of change is observed invariably in repeated impalements of the same specimen, so long as the cellular structure remains unaffected.

Kopac ('36) observed that the membrane resistance of the cell of *Valonia*, impaled with a wide electrode, increased gradually until it reached a high value. Possibly the appearance of the small potential difference at the very beginning of the measurement in *Noctiluca* is related to the early stage of healing around the electrode and, consequently, its magnitude should be considered as the potential difference between the cell sap and sea water across the thin protoplasmic layer. On the other hand, the potential values after the marked ascent may represent the resting potential between the protoplasm and the outer medium.

In some of the experiments, the resting potential gradually decayed after 10 to 20 minutes. In every such instance a vacuole was formed which separated the tip of the electrode from direct contact with the protoplasm. This decay and the small potential at the beginning of the measurement may possibly be explained as follows: the latter is an algebraic sum of two potentials of approximately the same magnitude but of opposite sign, one of which is the resting potential while the other is a potential difference which can be expected to exist at the boundary between the protoplasm and cell sap. Then it seems likely that the boundary of the vacuole at the tip also has a potential difference, which gradually increases according as the vacuolar sap becomes more similar in nature to the cell sap, and this potential cancels the measured resting potential. Samples of cell sap were squeezed out and examined with a flame spectrophotometer; the concentration of potassium ion in the cell sap was found to be approximately equal to that in normal sea water (8.9 mM in cell sap, 10.1 mM in artificial sea water). This fact agrees

well with the expectation of a potential difference at the interface between the protoplasm and the cell sap.

In these experiments the cells were mainly impaled at points considerably distant from the oral opening. In order to see whether the resting potential values varied according to the point of impalement, the electrode was inserted into various parts of the cell. No appreciable difference in values was found in any part of the cell so long as the tip of the electrode was in contact with the protoplasm. There was only a negligible potential difference led out from an electrode placed in the cell sap. However, an abrupt change in potential arose as the tip of the electrode passed through the cell sap and penetrated the nuclear mass.

In a majority of experiments, occasional rise and recovery of potential were observed. The maximum value of the rises was read as about 80 mV negative to the outer medium. It was thought that there might be some correlation between this fluctuation and the beating of the tentacle. As a rule the tentacle of *Noctiluca* moves quite freely but irregularly, sometimes pausing for long periods. At times when it is not showing spontaneous activity, active movement may usually be elicited by mechanical stimulation, as when its surface, or that of some other part of the cell, is touched by the electrode. However, the fidelity of the capillary electrometer to potential changes of high frequency is poor, and it was sometimes barely possible to correlate potential fluctuation with beating of the tentacle. It was decided therefore, to compile the average for the resting potential only from those values which had been taken when the tentacle was showing no perceptible movement. The details of action potential accompanying tentacular activity will be given in a later section.

As might be expected, the resting potential of *Noctiluca* is altered greatly when the external potassium concentration is changed. The cell, at first immersed in normal artificial sea water, was impaled with the electrode and kept standing until a stationary resting potential had been attained. Then the medium was replaced with a solution of different potas-

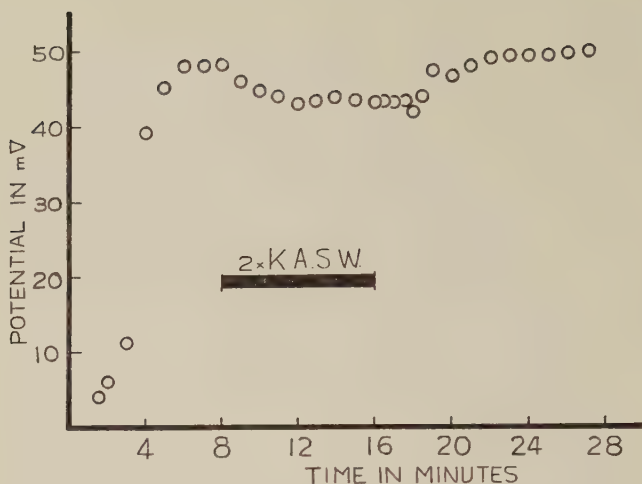


Fig. 3 Effect of artificial sea water containing 20.2 mM of potassium on the resting potential. The normal medium was replaced with the solution for the duration marked with solid rectangle. Temp. 19°C.

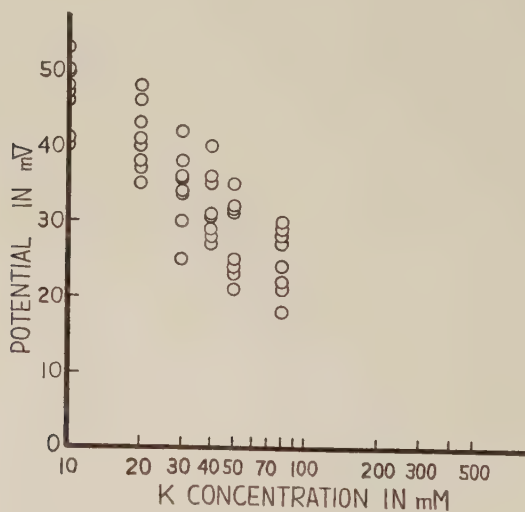


Fig. 4 Effect of increase in the potassium concentration outside on the resting potential. Potassium concentration is plotted in logarithmic scale. The values of resting potential depend greatly on the concentration gradient of potassium between outside and inside the protoplasm. Potassium concentration in normal artificial sea water is 10.1 mM. Temp. 21.0°C.

sium concentration. A representative result of this procedure is plotted in figure 3. In this case the potassium concentration in the medium was raised to twice as high as normal. The experimental solution was run into the bath through the water inlet at the time indicated on the figure. After 8 minutes the normal medium was reintroduced. Successive sets of experiments in which the potassium concentrations were varied from 20.2 mM to 70.7 mM were carried out on the same specimen. It will be seen that the resting potential begins to decrease within a few seconds after replacement of the solution, though the exact moment of exchange can not be pointed out with this method. The values for resting potential obtained in the normal medium after exposure to potassium rich solutions were actually lower than before but, in general, there was no significant difference between these potentials.

Eight successful experiments of the type shown in figure 3 were performed with solutions containing 20.2, 30.3, 40.4, 50.5, and 70.7 mM potassium. The results are summarized in figure 4 which illustrates the relation obtained when the values of resting potential are plotted against the logarithm of the concentration. It will be seen that the resting potential measured between protoplasm and sea water decreases markedly as the external potassium increases. An extrapolation of the data seems to cross the abscissa at a point near the isotonic potassium concentration, although the actual value of the potential at the isotonic concentration (550 mM) could not be obtained because of the toxic effect of potassium.

Action potential. The following experiments were performed using fine tip microelectrodes. Whereas it is relatively easy to insert such an electrode transversely into a muscle or nerve without causing appreciable damage it is difficult to do this through the pellicle of *Noctiluca*. The tip was often broken as it touched the pellicle, without the break being recognized.

The sequence of events which occurred following a successful impalement is shown in figure 5. When the microelectrode was lowered into position by means of the micro-

manipulator and was placed directly above a cell arrested under the microscope, the zero line was read as is shown in the left part of the record. Then the electrode was lowered further to impale the cell. A sudden change of potential appeared immediately after the electrode came in contact with protoplasm through the pellicle, though the microscopical observation provided no discernible indication of the moment when the electrode entered the cell. Owing to the rigidity of the pellicle in this instance, the electrode apparently pierced the thin protoplasmic layer. Thus, the marked change of potential suddenly disappeared. Following this there was

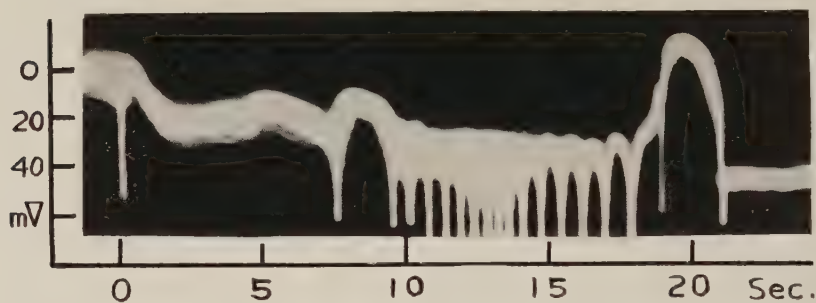


Fig. 5 Potential change recorded when the tip of microelectrode was introduced into the cell. Action potentials appeared correlating with the spontaneous activity of tentacle. Tip diameter of microelectrode is less than 0.5μ .

a rather slow return to the former potential value, probably caused by healing of the leak as described above. Usually, the resting potentials obtained with these fine electrodes appeared instantly and remained constant. They agreed well with those obtained potentiometrically. Furthermore, no appreciable potential difference was found between the cell sap and sea water across the protoplasmic layer. This, too, was in good agreement with what had been observed before.

The rest of the record shown in figure 5 shows action potentials caused by spontaneous tentacular activity. The most striking property of the action potential observed in *Noctiluca* is its hyperpolarization during the spike, that is, electro-

negativity of protoplasm increases during the spike instead of decreasing as in muscle, nerve and many other cells.

In many cases, considerable numbers of action spikes would occur in groups, with pauses between groups. After a pause where the resting potential was being resumed, the next group of action potentials ensued beginning with a small deflection of depolarization. This small depolarization was almost invariably observed before every action potential, therefore, the bases of consecutive spikes showed slightly lower values than the resting potential.

The average value of the action potential measured from the level of resting potential is 30 mV, while that of the resting potential is 45 mV. The resultant height of the action potential is 75 mV and that from the base of the small depolarization is 40 mV. On the contrary, some cases showed no appreciable pauses between the spikes. And a number of specimens showed neither action potential nor tentacular movement for long periods.

Through simultaneous observation of action potential and spontaneous tentacular activity it was found that an action potential spike is elicited at the very moment the tentacle begins to beat, and its height does not vary even if the beat is quite small in amplitude. A group of spikes corresponds to a whole deflection of the tentacle consisting of many such small beatings. Whenever an action potential appeared on the cathode ray tube a correlated beating of the tentacle was recognized without exception.

Although there was great individual difference in the frequency of the action potentials, values from 2 to 3 per second were maximum. In some cases, a large depolarization was observed after a group of spikes, and also the small depolarizations before individual spikes became larger according to the length of interval. In order to explain these depolarizations, further investigations will be required.

The action potential could be elicited by electrical stimulation. One more electrode was inserted and placed in the cell sap and through this a rectangular current was applied

across the very boundary of protoplasm of which the resting potential was being recorded. Some of the results are shown in figure 6. The deflection in the record indicates the direction as well as the strength of the stimulating current (upward: inside positive). The results suggest that the outward de-



Fig. 6 Stimulation of the cell with rectangular pulse applied through the polarizing microelectrode. Outward current elicited an action potential and, accordingly, a beating of tentacle.

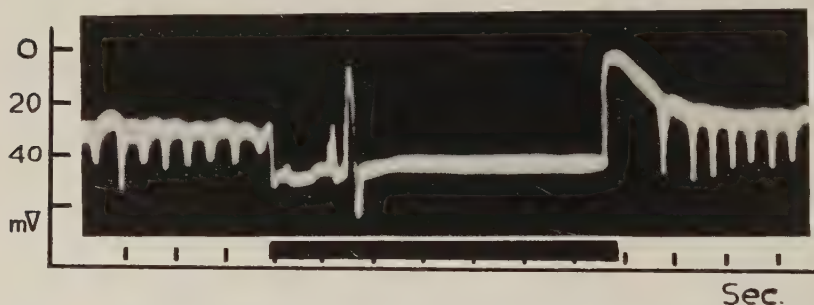


Fig. 7 Effect of increase in the internal negativity. An increase in negativity perfectly inhibited both the spontaneous electrical and tentacular activity. The duration of potential applied was indicated by solid rectangle on the time axis.

polarizing current is definitely effective while the inward current can not provoke the action potential.

Next, an attempt was made to observe modification of the spontaneous electrical activity by extrinsic potential difference across the membrane. While the spontaneous activity was led out from the protoplasm, known direct current was given through the polarizing electrode placed aside the

recording electrode. As shown in figure 7, the recorded negativity of the inside of the cell increases as the result of inward current applied. This shift of resting potential resulted in perfect inhibition of the spontaneous action potential as well as the tentacular activity. On the contrary, when an outward current was applied, the magnitude of spontaneous action potential did not undergo the change in height from the zero level (fig. 8). Moreover, on return to normal potential a kind of post-inhibition was frequently observed (right end of the record). In both cases no appreciable shifting of the resting potential followed after the applied current was cut off.

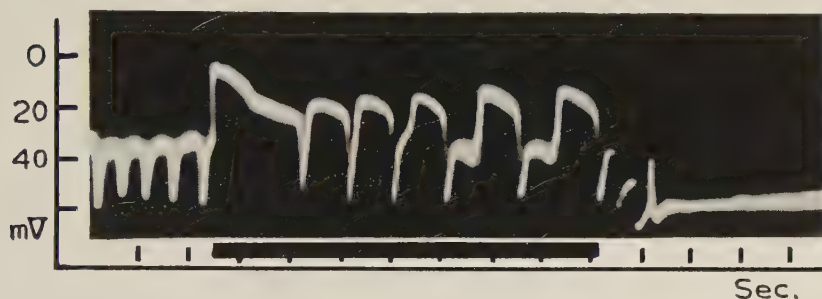


Fig. 8 Effect of decrease in the internal negativity. Total height of spikes did not vary though the frequency decreased. Note the post-inhibition of spontaneous activity (right end).

From these results it may be concluded that so far as the effect of electrical stimulation and tonus are concerned, the cell has no peculiarity, and has the same excitable properties as muscle or nerve cells, even though it shows a distinctive form of action potential.

As is well known, the bioluminescence of *Noctiluca* can be elicited by various stimuli. In the present investigation, touching by the holding device or the microelectrode usually evoked luminescence. Attempts were made unsuccessfully to detect the potential change at the time of light production.

The question arises as to whether the spike of the action potential could be merely a result of mechanical deflection of the electrode caused by beating of the tentacle. Actually,

beating of the tentacle sometimes did cause the cell to rotate. Moreover, it was confirmed that the potential attained when the electrode was bent by an artificial distortion invariably appeared as an increase of positivity at the tip. But in every case in which the action potential was recorded, the cell was firmly arrested and, therefore, the possibility of even a slight bending of the electrode according to the motion of the tentacle can be laid aside. Thus, there is no reason to think that the action potential can be attributed to any artefact resulting from the experimental arrangements.

Since the action potential observed is of opposite polarity to those of many other cells, it appears now to be impossible to interpret this phenomenon in the light of presently known facts.

SUMMARY

Resting and action potentials of *Noctiluca scintillans* were measured by means of internal electrodes. The resting potentials were obtained potentiometrically using electrodes having tip diameters of $2\ \mu$, as necessitated by measuring equipment. Oscillographic observation of action potentials was accomplished with electrodes having tip diameters of $0.5\ \mu$ or less.

The average resting potential was 48 mV with the wide electrode, and 45 mV with the fine electrode at room temperature.

When the cell was impaled with the wide electrode, which inevitably caused damage, the resting potential appeared after a few minutes.

The presence of a potential difference at the interface between the protoplasm and the cell sap was pointed out.

The resting potential was found to be greatly dependent upon the concentration of potassium in the medium outside. An increase in potassium concentration caused a marked and reversible decrease in the magnitude of the resting potential.

An action potential correlated with spontaneous activity of the tentacle was found. The most striking property of this action potential is hyperpolarization during the spike.

Oscillographic records showed that the magnitude of the action potential from the zero level is about 75 mV negative to the outside medium.

The effect of applied electric current on the spontaneous action potential and tentacular activity were studied. No particular differences in response as compared with muscle and nerve cells were found.

ACKNOWLEDGMENTS

The author is greatly indebted to Professor H. Kinosita for his kind guidance and encouragement throughout the experiments and for reading the manuscript.

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ADENOSINETRIPHOSPHATASE IN THE MITOCHONDRIA OF UNFERTILIZED AND NEWLY FERTILIZED SEA- URCHIN EGGS¹

A. MONROY

Laboratory of Comparative Anatomy, The University, Palermo, Italy

TWO FIGURES

On the occasion of the Symposium on the Biochemical and Structural Basis of Morphogenesis (Utrecht, '52) the present writer ('53) focused attention on the role that mitochondria may play in the process of the activation of the egg. It was suggested in particular that upon fertilization the removal of an inhibitor may be responsible for the activation of the mitochondrial enzymes.

It appeared then worth-while to examine the possibility that there may be a difference in the mitochondria isolated from unfertilized and newly fertilized eggs.

As a first approach to this problem a mitochondrial protease and the mitochondrial adenosinetriphosphatase (ATP-ase) have been studied and the results seem to lend support to the hypothesis that mitochondria undergo a physiological re-arrangement as a result of fertilization.

A temporary decline of protease-activity of the mitochondria has been shown to occur during the first 5 or 10 minutes following fertilization while at the same time there is a rise in protease-activity in the rest of the cytoplasm (Maggio, '57).

The results presented in this paper give evidence of an increased activity of the mitochondrial ATPase as a result of fertilization. Mullins ('49), using lyophilized sea-urchin eggs, had already found that the ATPase activity is nearly doubled

¹ This work has been aided by a Grant from Consiglio Nazionale delle Ricerche.

immediately after fertilization and that the activity could be accounted for almost entirely in the fraction insoluble in KCl, i.e. in the granules fraction. An increase of the ATPase activity in egg extracts after fertilization was also found by Connors and Scheer ('47). In both these investigations Ca^{++} was the only ion used as an activator.

METHODS

Eggs of *Paracentrotus lividus* were collected by letting the removed ovaries shed in sea-water; tissue debris were removed by filtering the eggs through cheese-cloth. After a few washings in sea-water, each batch of eggs was divided into two samples, one which was left unfertilized while the other was inseminated and collected for the experiment 3 to 5 minutes after the appearance of the first indications of fertilization (a slight shrinkage of the egg surface immediately followed by the elevation of the fertilization membrane). Samples in which less than about 95% of the eggs appeared to be fertilized were discarded.

The eggs were freed of their jelly-coat by a short treatment with acidified sea-water, collected by low-speed centrifugation and homogenized with about ten volumes of 0.44 M sucrose in 0.1 M citrate buffer pH 6.3 and Versene at the final concentration of 5.10^{-4} M, as suggested by Witter et al. ('55).

After a first centrifugation at low speed, the mitochondria were sedimented at 20,000 $\times g$ for 30 minutes in the cold. Washing of the mitochondrial pellet in 0.25 or 0.44 M sucrose results in a mitochondrial preparation still contaminated mainly with pigment. The appearance of the pellet is similar to that described by Laird et al. ('53) for the liver mitochondria, i.e. a more compact yellowish bottom layer and a reddish fluffy layer on top of it. However, if the pellet after the first high-speed centrifugation is re-suspended (making use of a homogenizer) in 1 M sucrose and centrifuged at high-speed for 30 minutes, the pigment forms a layer floating at the surface of the sucrose, while the mitochondria are sedimented as a slightly yellowish pellet. A similar procedure had already

been used by Monroy and DeNicola ('52) for the preparation of the pigment granules of the *Paracentrotus* egg. One or two further washings in 1 M sucrose usually suffice to obtain a tolerably clean suspension of mitochondria, as it is shown by the examination of the preparations in the electron microscope. For this purpose, samples of the preparations were suspended in 0.25 or 0.44 M sucrose in 0.01 M barbiturate buffer pH 8.0 and to each 1 cm³ of the suspension, 3–4 drops of a 2% solution of osmic acid were added and the mixture left overnight in the refrigerator; the preparations were shadowed with tungsten. They were examined and photographed in the Philips Electron Microscope in the Department of Hygiene, Medical School, the University of Palermo, kindly placed at our disposal.

For the study of the ATPase activity, the mitochondrial pellet was suspended either in 0.25 M sucrose in 0.01 M barbiturate buffer pH 8.0 or in 0.01 M barbiturate buffer pH 8.0 only (hypotonic medium). To ensure a homogeneous suspension of the mitochondria a small glass homogenizer with a teflon pestle was used. All the operations were performed at 2°C. The ATPase reaction was done in the final volume of 1.0 cm³ in a mixture containing barbiturate buffer pH 8.0, 0.02 M final concentration, sucrose 0.25 M final concentration 4 μ M of Ca⁺⁺ or Mg⁺⁺, 2 μ M of ATP, 0.2 cm³ of mitochondria suspension. When the reaction was to be carried out in hypotonic medium, distilled water was substituted for sucrose. The mixture was incubated at 30°C and the reaction was stopped by the addition of 1.0 cm³ of 10% trichloroacetic acid. By preliminary experiments it was shown that the spontaneous release of inorganic P from the mitochondria is negligible and in any case no differences were found between the mitochondria of unfertilized and newly fertilized eggs. For the determination of the inorganic P, preliminary assays showed that identical results were obtained either with the Lowry and Lopez ('46) or with Allen's ('40) method; thereafter in the majority of cases the latter was used. Total P was determined in the mitochondrial suspension and this was

taken as a reference for the activity of the ATPase. ATPase activity has been expressed as μg of inorganic P released from the ATP *per* 10 μg of total P in the mitochondrial suspension *per* 30 minutes of incubation at 30°C.

RESULTS

The mitochondria of the egg of *Paracentrotus lividus* are small, spherical bodies which stain blue with Janus Green B. Measurements of their diameter have been taken on the

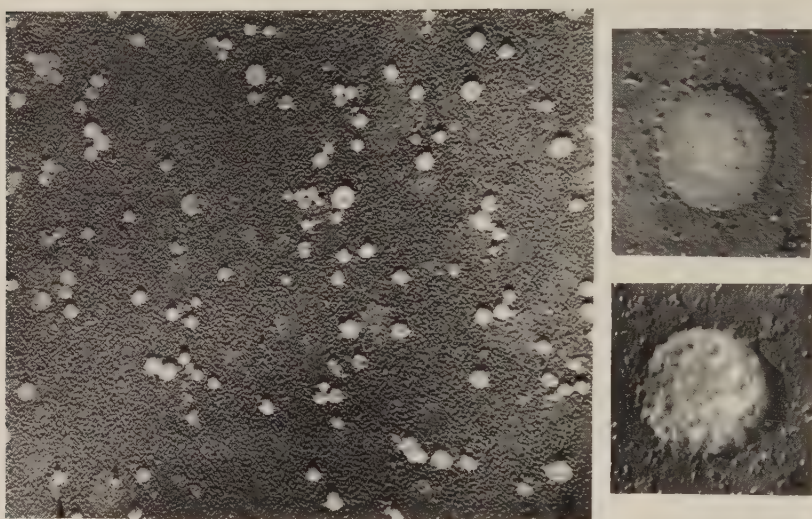


Fig. 1 Electron micrographs of mitochondria isolated from eggs of *Paracentrotus lividus*. (a) 4500 \times ; (b) and (c) 25,000 \times .

electron micrographs at different carefully checked enlargements (4,500 \times , 7,000 \times and 10,000 \times). Average values between 0.55 and 0.80 μ have been found. Allowance must be made for some degree of shrinkage during the preparation for the examination in the electron microscope (Fig. 1).

As to the ATPase activity, the following points seem to be worth mentioning.

1. In these experiments, Ca^{++} , at the concentration used, depressed the ATPase activity of the isolated mitochondria which, on the other hand, was activated by Mg^{++} (table 1).

The Magnesium-activation of the mitochondria ATPase described here is not a unique feature as it has been observed also in other cases (e.g. in the mitochondria of the Housefly Saktor, '52, '53) and in the liver mitochondria (Novikoff et al., '52). In connection with the inhibitory effect of Ca^{++} it is interesting to mention that the Magnesium-activated ATPase of muscle (Kielley and Meyerhoff, '51) has been found to be inhibited by Ca^{++} in the presence of Mg^{++} . It may be suggested that in the present case the inhibition of the ATPase caused by

TABLE 1

ATPase activity of mitochondria from unfertilized and newly fertilized eggs of Paracentrotus lividus and the effect of Ca^{++} and Mg^{++} . Activity in micrograms of inorganic P released from the ATP per 10 μg of total P in the mitochondria preparation after 30 minutes of incubation at 30°C.

| EXP. NO. | MEDIUM | UNFERTILIZED | | | 5 MINS. AFTER FERTILIZATION | | |
|-------------|-------------|-------------------|------------------|------------------|--------------------------------|------------------|------------------|
| | | No acti- vator | Ca^{++} | Mg^{++} | No acti- vator | Ca^{++} | Mg^{++} |
| 10 | Sucrose | 2.5 | | 6.57 | 2.2 | | 7.5 |
| 13 | Sucrose | 5.5 | 3.0 | 12.8 | 5.9 | 2.1 | 16.4 |
| 15 | Sucrose | 1.97 | 0.9 | 3.29 | 2.14 | 1.57 | 5.86 |
| 22 | Sucrose | 2.33 | 1.92 | 3.9 | 3.18 | 2.36 | 3.9 |
| 24 | Sucrose | 3.5 | | 6.9 | 4.38 | 2.7 | 8.44 |
| 11 | Dist. water | 4.17 | 1.97 | 10.0 | 2.97 | 2.18 | 12.9 |
| 12 | Dist. water | 3.43 | 1.65 | 7.63 | 4.17 | 3.3 | 8.5 |
| 14 | Dist. water | 2.22 | 0.33 | 4.77 | 3.71 | 2.14 | 6.57 |
| 17 | Dist. water | 6.17 | | 8.62 | 8.16 | | 10.6 |

Ca^{++} may be due to some high Magnesium content of the mitochondria. (Liver mitochondria have been found in fact to contain appreciable quantities of Magnesium, Siekewitz and Potter, '55).

2. ATPase activity is enhanced in hypotonic media. In these the conditions of permeability of the mitochondrial membrane are likely to be altered. This result is in accordance with the view that in the mitochondria the ATPase is present in a latent condition (Kielley and Kielley, '42) and its activity is enhanced, among others, by aging. In the present experi-

ments, aging has given variable results (table 2); this point, however, needs further examination.

3. The activity of the mitochondrial ATPase undergoes an increase during the first few minutes after fertilization (tables 1 and 2). This becomes especially evident in the mitochondria suspended in hypotonic media. In some experiments in fact the value obtained with mitochondria in sucrose was identical before and after fertilization. But when the activity of the same preparation was tested in hypotonic medium then the mitochondria of the fertilized eggs exhibited a higher activity.

TABLE 2

ATPase activity in the mitochondria of the unfertilized and fertilized eggs of Paracentrotus lividus in sucrose and in distilled water and the effect of aging. Activator: 4 μ M Mg⁺⁺.

| UNFERTILIZED | | | | FERTILIZED | | | |
|--------------|----------------|---------|----------------|------------|----------------|---------|----------------|
| Fresh | | Aged | | Fresh | | Aged | |
| Sucrose | Distill. water | Sucrose | Distill. water | Sucrose | Distill. water | Sucrose | Distill. water |
| 5.48 | 7.55 | 5.70 | 7.17 | 6.24 | 8.40 | 6.1 | 6.3 |
| 3.97 | | 5.62 | | 3.90 | | 3.70 | |
| 3.29 | 4.77 | | | 5.86 | 6.57 | | |
| 4.86 | 6.44 | 3.60 | 4.58 | 5.30 | 8.70 | 7.12 | 7.53 |
| 3.98 | 6.70 | 3.17 | 4.56 | 3.94 | 8.52 | 2.94 | 7.10 |

A typical experiment is illustrated in the diagram figure 2. In this experiment the ATPase activity was tested at different time intervals for a period of two hours, on two mitochondrial preparations from the same batch of eggs, one unfertilized and the other fertilized, in sucrose and in distilled water. The higher activity of the preparation of mitochondria from newly fertilized eggs, especially in those suspended in distilled water, is quite clear.

It must be added at this point that in some experiments, carried out during the winter season, the mitochondria of the fertilized eggs yielded lower ATPase activity than those of the unfertilized eggs. In these cases, even when the reaction was carried out in hypotonic media, the activity remained

lower than in the unfertilized eggs. No satisfactory explanation can be offered of this result at the moment, except that some damage may have been caused during the preparation to particularly fragile mitochondria.

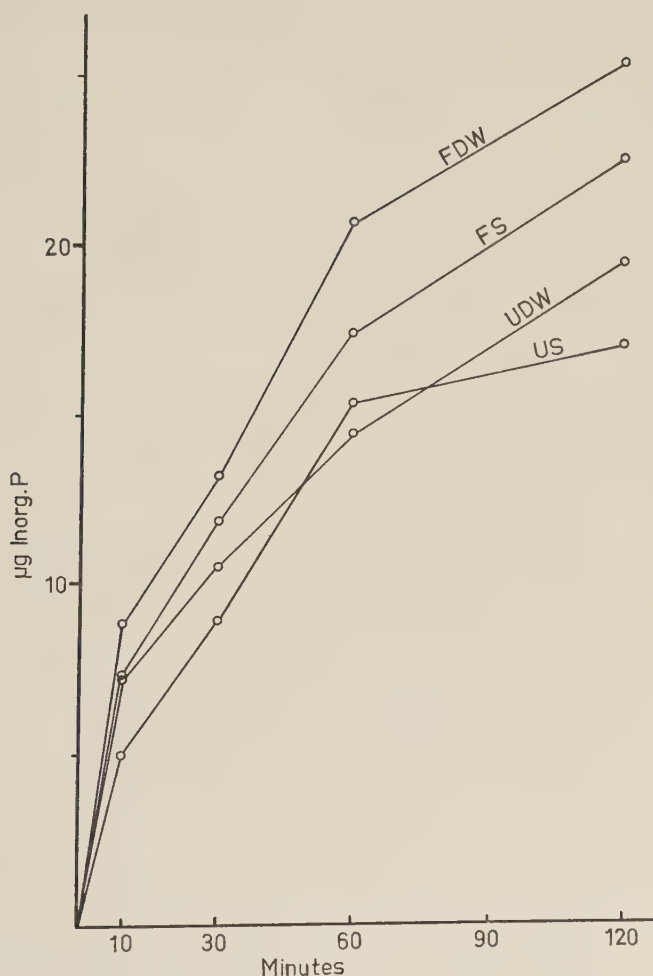


Fig. 2 ATPase activity in mitochondria of unfertilized and newly fertilized eggs of *Paracentrotus lividus*. Experimental conditions as specified in text. US = mitochondria from unfertilized eggs in sucrose; UDW = the same preparation in distilled water; FS = mitochondria from newly fertilized eggs in sucrose; FWD = the same preparation in distilled water.

These results, together with those on the mitochondria protease (Maggio, '57), supply a first direct evidence that the mitochondria of the sea-urchin egg undergo a physiological and most probably structural re-arrangement immediately following fertilization. They also support the view (Monroy, '53) that activation of the mitochondria is one of the events of the activation of the egg.

SUMMARY

1. A Mg^{++} -activated ATPase is present in the mitochondria of both unfertilized and newly fertilized eggs of *Paracentrotus lividus*.

2. The activity is higher in mitochondria suspended in a hypotonic than in a supposedly isotonic medium (0.25 M sucrose).

3. A rise of the ATPase activity is shown to take place during the first few minutes following fertilization.

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THE ACTION OF ULTRASOUND ON THE MOUSE LIVER ¹

EUGENE BELL ^{2,3}

*Arnold Biological Laboratory, Brown University,
Providence 12, Rhode Island*

SEVEN FIGURES

INTRODUCTION

When the mouse liver is irradiated with ultrasound of high intensity a part of the tissue is necrotized. If only a small volume of tissue in the intact mouse is irradiated, the well-being of the animal is not seriously affected and the sequence of changes in the liver can be studied over a long period. The present report is concerned with some cytological changes in liver tissue damaged by ultrasound of one or 27 megacycles frequency, and with the physical events involved in these changes. It is also concerned with liver repair following injury with ultrasound.

The necrotizing effect of ultrasound on the liver has been reported by a number of investigators (Koeppen, '49; Bell, '53; Southam, Beyer and Allen, '53; and Dönhardt and Presch, '53), but neither the histological events which culminate in necrosis, nor the course of necrosis itself, has been studied in detail. That the special character of the necrosis may be responsible for delaying the onset of mitotic activity in mouse livers damaged with ultrasound has been suggested in a preliminary report (Bell, '53). In the present study it is confirmed that the interval between injury and the onset

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² Post-Doctoral Fellow, 1954-56; United States Public Health Service.

³ Present address: Biology Department, Massachusetts Institute of Technology.

of mitosis is significantly longer in livers damaged with ultrasound or cautery than it is in livers damaged by ordinary injurious agents.

Lehmann ('53) has suggested that the heating action of ultrasound is primarily responsible for tissue damage produced by irradiation. However, Fry et al. ('50) found that damage to nerve tissue could not be accounted for by heating which occurs during the application of ultrasound. Goldman and Lepeschkin ('52) have reported that cytological changes in a variety of biological materials exposed to ultrasound were different from changes produced by heating alone. The results of the present investigation indicate that the average thermal change which occurs in pre-cooled liver tissue irradiated with ultrasound cannot account for cytological changes which are observed following treatment. Cytological changes essentially like those observed in livers irradiated with ultrasound are found to occur, however, when the liver is exposed to concentrated radiant heat. These changes occur if the average thermal change in the liver during exposure to concentrated radiant heat is 15°C. greater than that during exposure to ultrasound. Local heating however cannot be ruled out as a factor which may be responsible for damage.

MATERIALS AND METHODS

Two ultrasonic generators built in the Metals Research Laboratory of the Graduate Division of Applied Mathematics at Brown University provide a range of frequencies between one and 27 megacycles. One and 9 megacycle quartz crystals are used as transducers, the latter being driven at its third harmonic. The experimental arrangement employed for irradiating livers in intact mice is shown in figure 1. Crystal holders are mounted in the end wall of a 10 gallon tank filled with degassed distilled water which serves as a coupling medium for the sound. A planoconcave polystyrene lens [Bronzo and Anderson, '52] used to focus the output from the one megacycle transducer is inserted into a recess of the crystal holder and is separated from the crystal by a layer of degassed

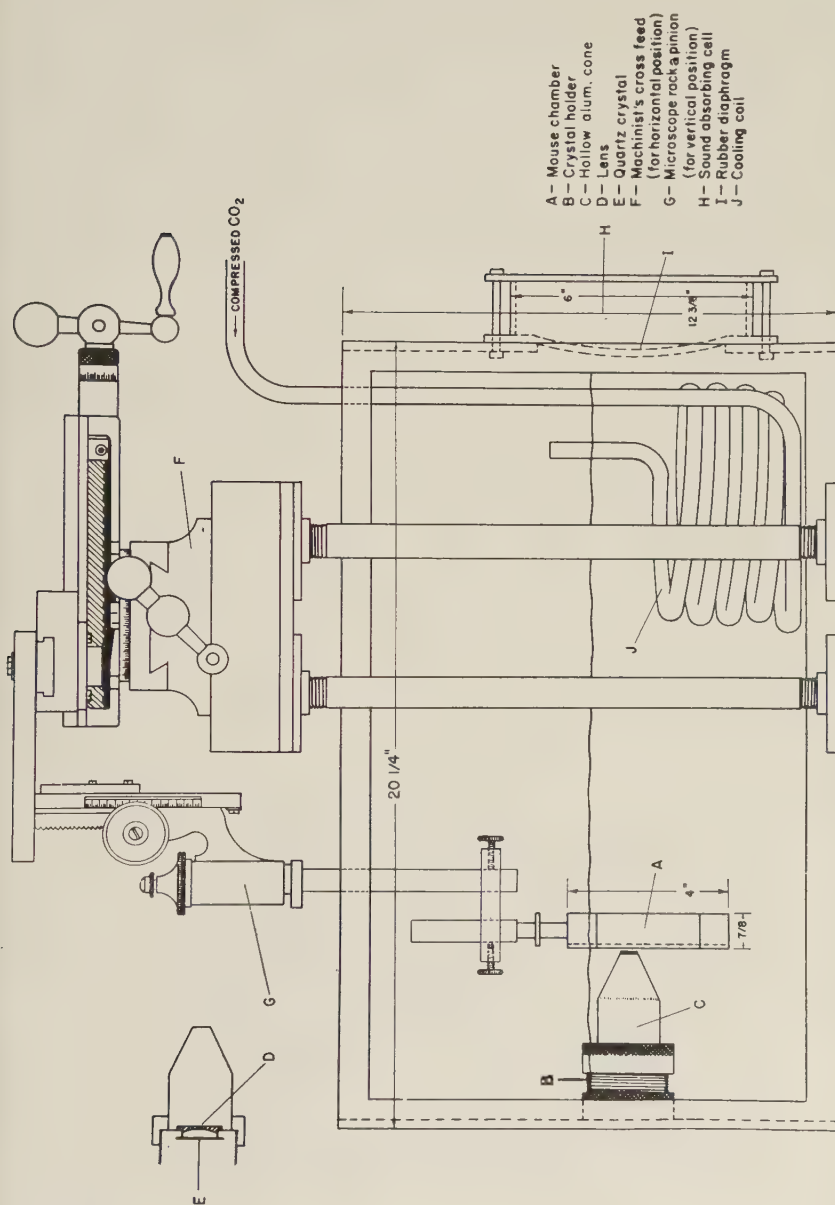


Fig. 1 Experimental arrangement for irradiating intact mouse liver with ultra-sound. Leads from generator are connected to terminals on the crystal holder (not shown).

distilled water 0.64 cm deep. The lens has a 2.56 cm radius of curvature and is ground to a center thickness of 1.27×10^{-2} cm. By using a lens it is possible to irradiate a small volume of tissue at high acoustic intensity. The truncated apex of a hollow aluminum cone threaded over the crystal holder marks the location of the focal region of the lens and thereby facilitates positioning of an animal in the focus of the sound beam. For irradiation with ultrasound of 27 megacycles frequency, animals are positioned 0.8 cm from the transducer and irradiated through a small aperture cut in the skin overlying the liver. At 27 megacycles one-half the energy from the transducer is absorbed in less than 1.5 cm of aqueous medium, hence it is not feasible to use focused sound at this frequency.

Power output at one megacycle is measured with a Sieman's Sonotest meter and at 27 megacycles with a calorimeter.

Experimental animals were 3 to 4 month old BUB and BUC white mice bred at Brown University. Animals are prepared for irradiation by shaving the hair on the abdomen. The liver can then be seen below the translucent overlying tissue. Animals are anesthetized with ether and placed in a plastic chamber shown in figure 2. An animal is immobilized in the

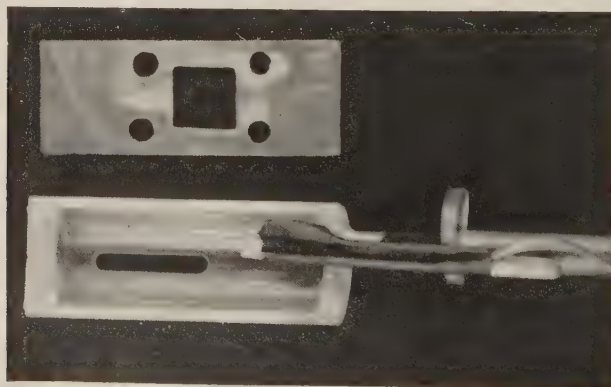


Fig. 2 Plastic chamber used for holding mouse during irradiation with sound or heat. Thermocouple is embedded in hypodermic needle.

chamber by placing its legs through holes in the cover piece; the legs are then taped to the chamber with Scotch electrical tape. A rectangular cutout in the cover of the chamber lies over the region of the upper abdomen so that that portion of the animal is in direct contact with water when the chamber is placed in the bath. The chamber is clamped to the post of a three coordinate positioning system and the liver is aligned with the apex of the aluminum cone so that it will lie in the focal region of the sound field. The duration of exposure is controlled by an external timer connected to the screen of the oscillator tube.

A 100 watt projection lamp mounted in a spherical illuminator was used as a source of radiant heat. Light from the illuminator was focused onto the exposed left lateral lobe of the liver with a lens of 2 cm diameter and 3.28 cm focal length.

Histological procedure: Animals are killed by a blow on the head, decapitated and thoroughly bled. Tissue is taken immediately and is fixed in Bouin's fluid for staining with hematoxylin and eosin; in Rossman's fluid for the McManus demonstration of glycogen with the Schiff reagent; or in neutral formalin for demonstration of fat with Sudan IV. The material is oriented for sectioning so that it can be cut in a plane which is perpendicular to the ventral surface of the lobe. The cylinder of tissue traversed by sound is thereby revealed in longitudinal section.

RESULTS

The location of zones of necrosis produced by focused ultrasound of one megacycle frequency

When the liver is irradiated through the ventral skin with an output of between 1.5 and 50 watts (total power delivered to the lens), damage which leads to necrosis may occur at either the ventral or the dorsal surface of the target lobe. In general when livers are irradiated with sound of 20 watts

power or less, damage occurs at the lobe surface which is further from the sound source, that is, at the dorsal surface. If damage occurs at the dorsal surface of the lobe, liver tissue nearer to the sound source than the zone of damage is traversed by sound but not necrotized. This effect is shown schematically in figure 3. When two or three lobes, lying one behind the other, intercept the sound beam, damage occurs at the dorsal surface of each of the lobes. The zone of damage at the dorsal surface of the lobe furthest from the sound source is always smaller than the zone of damage in the lobe nearest to the sound source.

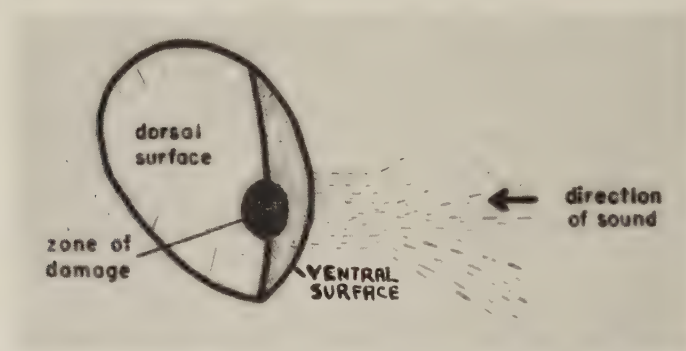


Fig. 3 Diagram showing location of zone of damage at dorsal surface of lobe when the liver is irradiated ventrally with focused ultrasound of one megacycle frequency. Stippled section is a plane through the zone of damage perpendicular to the lobe surface.

Liver necrosis produced by ultrasound of one or 27 megacycle frequency and changes which lead to it

Fifty-two animals were irradiated at one megacycle and 24 were irradiated at 27 megacycles. Animals were killed at intervals after irradiation as shown in table 1. At one megacycle, mice were exposed for 15 seconds to an output of 40 watts delivered to the first face of the polystyrene lens. At 27 megacycles, mice were irradiated with unfocused sound through a small aperture cut in the skin overlying the liver. They were irradiated for 15 seconds at an intensity of 35

watts/cm². When animals were killed, the gross appearance of the liver was recorded and liver tissue was taken for histological examination.

Gross appearance: Immediately following irradiation at one megacycle the damaged region of the liver, measuring 2 to 4 mm in diameter, is markedly darker than the surrounding tissue. The affected tissue appears congested. By 24 hours the tissue is completely blanched and remains so until it is sloughed sometime between 13 and 15 days. A visible depression marks the region from which the necrotic tissue separates. The only difference between the gross appearance of tissue irradiated at 27 megacycles and that irradiated at one mega-

TABLE 1

Schedule for serial sacrifice of animals following irradiation with ultrasound of one or 27 megacycles frequency

| TIME OF SACRIFICE AFTER IRRADIATION | NO. ANIMALS | | TIME OF SACRIFICE AFTER IRRADIATION | NO. ANIMALS | |
|--|-------------|-------|--|-------------|-------|
| | 1 mc | 27 mc | | 1 mc | 27 mc |
| Immediately | 6 | 5 | 6 days | 2 | |
| 1 day | 8 | 6 | 7 days | 2 | |
| 2 days | 4 | 3 | 8 days | 2 | |
| 3 days | 5 | 4 | 11 days | 2 | |
| 4 days | 4 | 3 | 14 days | 2 | |
| 5 days | 6 | 3 | 15 days | 9 | |

cycle is that following irradiation at the higher frequency: (1) a somewhat larger region is damaged and (2) the core of the necrotized tissue is colored green. The sequence of gross changes is otherwise the same. Histological appearance: Immediately following irradiation at one megacycle, sinusoids in the irradiated region are two to three times larger than normal, while following irradiation at 27 megacycles they are from two to 6 times larger than normal (fig. 4). The distended sinusoids are filled with erythrocytes. Sinusoids are markedly enlarged in the region which becomes necrotic as well as in a few cell layers adjacent to the zone of necrosis.

Macrophages which appear dislodged from their attachment to the walls of the sinusoids can be observed in the region

which is congested. This effect has been observed following irradiation at 27 megacycles but not after irradiation at one megacycle, and occurs only in those sinusoids which are greatly enlarged.

Vacuolization in parenchymal cells is more extensive following treatment with the higher frequency. The pattern of its occurrence is irregular regardless of frequency, that is, some cells appear vacuolated and others do not. However within parenchymal cells which are vacuolated, vacuoles occur pri-

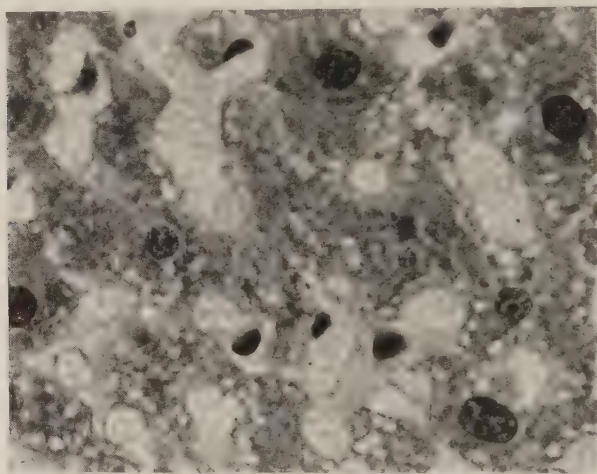


Fig. 4 Distended sinusoids filled with erythrocytes immediately following irradiation with ultrasound of 27 megacycles frequency. $\times 594$.

marily along cell surfaces which are contiguous to sinusoids and larger blood vessels. In sections stained with Sudan IV or with the Schiff reagent, numerous vacuoles were observed which contained neither fat nor glycogen. Cavities which correspond to "pseudo-cavitation holes" described by Hug and Pape ('54) were observed in some livers irradiated at one megacycle (fig. 5) but not in livers irradiated at 27 megacycles. The cells surrounding the cavity appear torn and their nuclei are pyknotic.

The most severely damaged liver cell nuclei are moderately hyperchromatic immediately following irradiation at one megacycle. Structural modification of both hyperchromatic nuclei and nuclei which stain normally appears to be related to the extent of cytoplasmic vacuolization. In regions where vacuoles are numerous some nuclei are deformed by adjacent vacuoles which seem to press against the nuclear membrane. The same effect has been observed in livers irradiated at 27 megacycles frequency, however in addition to structural de-

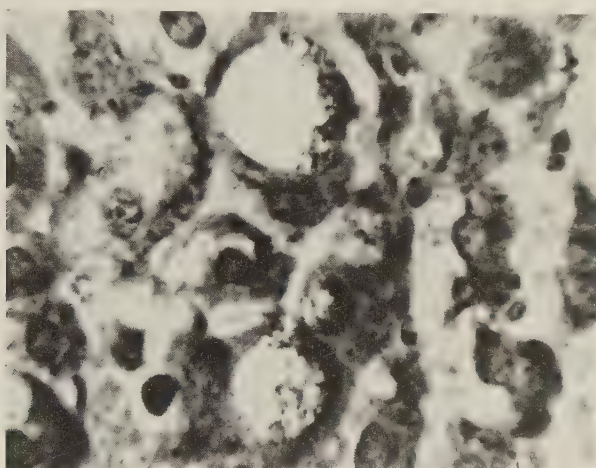


Fig. 5 "Pseudo-cavitation holes" in liver tissue immediately following irradiation with ultrasound of one megacycle frequency. $\times 594$.

formation, some nuclear membranes are ruptured. In some parenchymal cells in which the nuclear membrane is ruptured, the contents of the nucleus appear spilled out into an adjacent vacuole.

Further evidence of the disruption of cells in liver tissue is the occurrence of glycogen in lumens of small vessels and sinusoids in the zone of damage.

Not only are materials released into the circulation as a consequence of irradiation, but elements from the circulation are forced into liver cells. In particular, erythrocytes appear to have penetrated some parenchymal cells. This effect has

been observed following irradiation at either frequency (fig. 6). Most of the cytoplasm of such cells seems to have been displaced but the cell boundaries appear to have remained intact. Where this phenomenon occurs in a large number of cells in the same region of the liver, the cells are necrotized.

The foregoing effects have all been observed in liver tissue which is removed and fixed immediately following irradiation, and occur in the volume of tissue which will become necrotic. By 6 hours following irradiation the zone of necrosis is

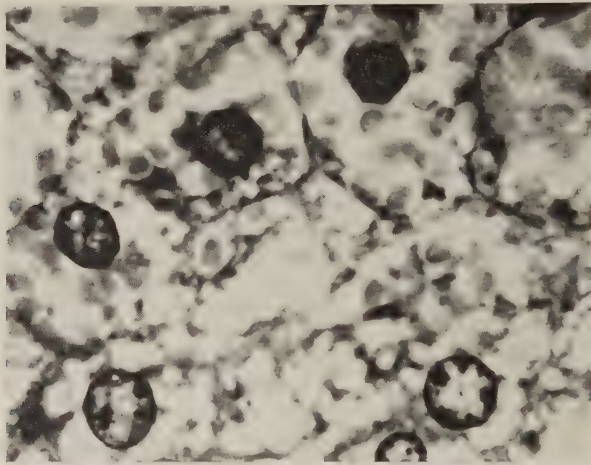


Fig. 6 Erythrocytes in liver cells immediately following irradiation with ultrasound of one megacycle frequency. $\times 1125$.

moderately well defined. In H and E preparations the cytoplasm of necrotic cells appears eosinophilic and nuclei are slightly hyperchromatic. By 24 hours the cytoplasm of necrotized cells is markedly eosinophilic (fig. 7). The necrotic tissue has been invaded by leucocytes, some of which accumulate at the boundary between the necrotized and living tissue. Most nuclei in dead cells still stain well but are reduced in size.

By 72 hours there is a noticeable increase of leucocytes within the necrotic tissue and these cells are more abundant at the boundary between the necrotic mass and the sublethally

irradiated cells. In surviving cells adjacent to the boundary there are a few mitotic figures. At 72 hours they are not found elsewhere in the liver.

After 4 days, nuclei in the necrotic cells are still stainable, and connective tissue has begun to appear at the boundary of the zone of damage. At this time cells adjacent to the necrotic mass are enlarged. No conspicuous changes occur between 5

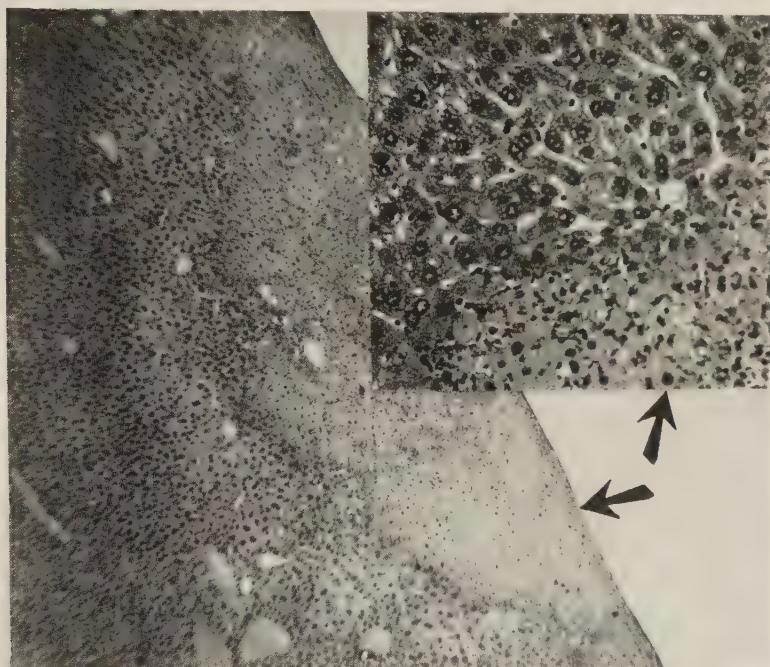


Fig. 7 Zone of necrosis 24 hours following irradiation with ultrasound of one megacycle frequency. Arrows point to necrotic tissue. $\times 35$ and $\times 125$.

days and 15 days when the necrotic mass is finally sloughed into the abdominal cavity. The dead material continues to be enveloped by connective tissue and the structural elements within the zone of necrosis fade gradually. As late as 11 days after injury, nuclei still stain and cell boundaries are discernible. When the plug of necrotic material is cast off between 12 and 15 days, a depression lined with connective tissue marks its place.

The initiation of mitotic activity following injury to the liver by ultrasound, heat and ligation

To determine whether the interval of delay between the time of injury and the onset of mitosis in the liver is related to the mode of injury, 58 animals were sacrificed at three days, 4 days and 5 days following damage to the liver by ultrasound, partial cautery or partial ligation. The livers of 27 animals were irradiated with ultrasound of one megacycle frequency, and 9 were irradiated at 27 megacycles. The procedure, doses and exposure time were the same as those described in the previous section, with the exception that between 5 and 50% of the left lateral lobe was damaged. The extent of damage was varied by moving the animal in the sound beam during irradiation. Following laparotomy 50% of the left lateral lobe was cauterized. Heat damage was inflicted with a hot glass probe applied directly to the surface of the lobe. The livers of 11 mice were damaged by cautery. Partial ligations were performed in 11 additional animals. To determine whether the time of initiation of mitosis depends upon the extent of damage done to the liver, in two animals 5% of the left lateral lobe was ligated, in two animals 10% was ligated and in 7 animals 50% was ligated. Broad dental tape was used to tie off portions of the left lateral lobe. The tape was drawn around the lobe as tightly as possible without cutting the lobe.

Following ligation, no matter how small the amount of tissue tied off, mitotic activity was observed by 72 hours in cells distributed throughout the unligated portion of the liver. In livers treated with heat or ultrasound only a few cells immediately peripheral to the zone of damage were found to be in division at this time. Elsewhere in the liver there was no mitotic activity. In cauterized livers it was not until 4 days after injury that dividing cells were found distributed throughout the entire liver and in livers irradiated at either one or 27 megacycles, mitotic figures were not generally distributed until the 5th day.

Comparison of cytological changes caused by cautery or focused radiant heat with those caused by ultrasound

To determine whether cytological changes caused by ultrasound also occur in livers exposed to heat alone, liver injury due to ultrasound was compared with liver injury due to various forms of heating. Two pairs of animals were used to determine cytological changes in the liver immediately after the direct application of heat. Livers of the first pair were injured with a needle probe flamed to a red heat. The probe was introduced into a lobe to a depth of about 1.5 mm, held in place for 5 seconds, and removed. Livers of a second pair were damaged with a flat glass probe also flamed to a red heat and applied to the surface of the lobe. Animals were anesthetized with nembutal prior to the operation and sacrificed immediately after it. Livers of 10 animals were treated with concentrated radiant heat. Of these, 6 were irradiated for 15 seconds at an intensity which produced a 15°C . rise in temperature in the liver, and 4 were irradiated for the same period, but the temperature rise in the liver during treatment was 35°C . Three animals of the first group and two of the second were sacrificed 24 hours after exposure; all others were sacrificed immediately after exposure. Details of the method of temperature measurement are given on page 96 of "Results."

Comparison of cytological changes in heat-treated livers with those in livers treated with ultrasound indicates the following: 1 — In livers damaged with a needle probe flamed to a red heat, cytological changes were in no way similar to those produced by ultrasound. 2 — In livers damaged with a flat glass probe, enlargement of sinusoids, apparent separation of macrophages from the walls of markedly enlarged sinusoids, hyperchromatic parenchymal cell nuclei and a small degree of vacuolization were observed. Although these phenomena also occur in livers irradiated with ultrasound, the following changes do not: stretched and separated cords of liver cells, obliteration of cell boundaries, and disappear-

ance of cytoplasmic basophilia. 3 — In livers irradiated with focused radiant heat which produced a thermal change of 15°C . in 15 seconds, slight enlargement of sinusoids, hyperchromatic nuclei and penetration of erythrocytes into parenchymal cells were observed. 4 — Finally in livers irradiated with focused radiant heat which produced a thermal change of 35°C . in 15 seconds, all of the cytological changes, with one exception, observed in livers treated with ultrasound were found to occur. "Pseudo-cavitation holes" were not observed.

Relation between heating and damage in liver tissue irradiated (a) with ultrasound and (b) with concentrated radiant heat

The temperature rise in liver tissue during irradiation with focused ultrasound of one megacycle frequency was measured in 15 animals for exposures of 15 seconds and in 6 animals for exposures of 45 seconds. Of the 15 animals irradiated for 15 seconds, 7 were cooled prior to irradiation so that the maximum temperature in the liver during irradiation did not exceed 36°C . The power output used was 40 watts delivered to the first face of the polystyrene lens. A copper-constantan thermocouple embedded in a 20 gauge hypodermic needle was connected with a high speed recording potentiometer so that a continuous record of temperature change during irradiation could be obtained. After an animal is anesthetized with nembutal the needle is inserted into the liver in the region to be irradiated. The irradiation procedure is the same as that described above (page 86).

When livers were irradiated for 15 seconds, the average temperature rise in the liver was 15°C ., while in tissue irradiated for 45 seconds the average temperature rise was 22°C . It was observed that during irradiation for 15 seconds the temperature rose steadily for the duration of the exposure. The rate of change of temperature under these conditions is then approximately 1°C . per second. Liver damage was observed in all 7 animals which were pre-cooled as well as in all 14 animals which were not pre-cooled.

The livers of 16 additional animals were irradiated with concentrated radiant heat. Following anesthetization with nembutal, the left lateral lobe of the liver was drawn out through an incision 1.0 cm long made in the abdominal wall. An area measuring 5 mm in diameter was exposed to focused light for 15 seconds. The temperature changes in the liver during treatment were measured in the same manner as above. The livers of 12 animals were irradiated with light of an intensity which produced a thermal change of 15°C. in 15 seconds. Of these animals 6 were cooled in an ice-bath prior to irradiation so that the maximum temperature during irradiation did not exceed 35°C. The livers of 4 additional

TABLE 2

Occurrence of damage in livers treated with radiant heat

| TEMP. RISE IN 15 SEC. | TEMP. MAXIMUM | NUMBER OF ANIMALS | |
|--------------------------|------------------|-------------------|-----------|
| | | Damage | No damage |
| 15°C. | 45°C. | 6 | 0 |
| 35°C. | 61°C. | 4 | 0 |
| 15°C. | 35°C. | 1 | 5 |

animals were irradiated with light of an intensity which produced a thermal change of 35°C. in 15 seconds. Five animals were sacrificed immediately following irradiation and 11 animals were sacrificed 24 hours after irradiation. The temperature rise, the maximum temperature during irradiation and the incidence of damage are shown in table 2. While damage was produced in all livers in which the temperature during irradiation rose to a point above normal body temperature, it was produced in only one liver when animals were pre-cooled.

It is apparent from these results that the liver can be protected, by previous cooling, against injury due to concentrated radiant heat even though the amount of energy and the rate at which it is applied is the same as that applied when the liver is not cooled. The foregoing results may be contrasted with those obtained when the liver is cooled prior

to irradiation with ultrasound. Pre-cooling does not protect the liver against damage from ultrasound. Although the average temperature in the liver during irradiation with ultrasound did not exceed normal body temperature, liver damage was still observed in all of the animals irradiated. Hence the average temperature change which takes place in pre-cooled livers during irradiation with ultrasound cannot explain the occurrence of necrosis.

The rate of change of temperature during irradiation with radiant heat was the same as that which occurred during treatment with sound, but no damage was observed in livers exposed to radiant heat if animals were pre-cooled.

DISCUSSION

The character of the necrosis produced in the liver following irradiation with ultrasound, although different from cell death produced by other injurious agents, resembles in some details the necrosis produced by ligation, and is strikingly similar to necrosis produced by focused radiant heat. Tissue necrotized by ultrasound or by ligation persists for 5 days or longer virtually unchanged, and its structural features fade only gradually after that period. This resistance to autolysis can be contrasted with the rapid and extensive dissolution of damaged tissue which occurs after carbon tetrachloride necrosis. As early as 24 hours following administration of CCl_4 , autolysis has already resulted in the disappearance of nuclei and cell boundaries. By five days, not only is the debris removed, but repair is almost completed. On the other hand, in tissue necrotized by ultrasound, by ligation, or by radiant heat, structural elements are preserved for a period longer than 5 days.

The gross appearance of tissue damaged by ultrasound 6 hours after irradiation shows that the affected region has already begun to blanch. The effect is probably an outcome of vascular occlusion. The presence of glycogen granules in

the blood, and of erythrocytes in parenchymal cells indicates that vascular walls may be damaged as a result of treatment with ultrasound. These events may trigger clotting in the zone of damage and lead to vascular occlusion. That blood flow into the region which becomes necrotic is cut off virtually instantaneously, is clearly demonstrated when Thorotrast is administered intravenously immediately following irradiation (Bell, '55). Thorotrast does not penetrate the zone of tissue which is most severely damaged. It appears that vascular occlusion, an expected consequence of ligation, and perhaps the result of coagulation of blood following irradiation with ultrasound or radiant heat, leads to necrosis which resists autolysis. It also appears that autolytic activity as evidenced by the breakdown of cellular structures depends to some extent upon blood flow through the zone of necrosis. The fact that blood flow is not arrested in CCl_4 necrosis perhaps explains the rapid rate of tissue dissolution, whereas tissue isolated from the blood circulation by ligation, ultrasound or radiant heat autolyses at a much slower rate.

Damage produced by ultrasound or by ligation is histologically similar in that structural breakdown is retarded in tissue necrotized by these means. Physiological changes which attend each of these types of injury may nevertheless differ. After ligation or after chemical or surgical damage to the liver, mitotic activity is generally apparent within two days in unaffected parts of the organ; and three days after damage mitosis is invariably present. On the other hand generally distributed mitotic activity was never observed at three days in livers damaged with ultrasound or in livers which were partially cauterized. Cell divisions are not observed throughout the liver until the 5th day after damage with ultrasound and not until the 4th day after damage by cautery. By the 5th day after CCl_4 injury, mitotic activity has practically if not completely ceased.

That mitosis is not evoked after the usual two to three day interval, following damage to the liver with ultrasound cannot be attributed to the small volume of tissue injured. It

has been shown that the mitotic response can be elicited by ligation after the usual delay even when the extent of damage is small. Ligation of 5, 10 and 50% of the left lateral lobe evoked mitotic activity by 72 hours in all livers studied.

Cell divisions were observed in ligated livers two to three days earlier than in irradiated livers even though retarded lytic activity was characteristic of both types on injury. Perhaps then, the delay in the onset of mitotic activity following injury with ultrasound or cautery cannot be attributed to the retarded rate of lytic activity. It might however be due to biochemical events within tissue necrotized by ultrasound or cautery which do not occur in tissue damage by ligation. It is suggested that events within the portion of liver which is necrotized, influence the process by which mitosis is initiated. It is also suggested that the initiation of mitosis cannot be explained solely in terms of a compensatory response of the undamaged parenchyma to "overwork." If such an explanation held, the interval between injury and the onset of mitosis would always be the same regardless of the way in which damage is inflicted. This is contrary to the evidence presented.

The close similarity between liver tissue damaged by ultrasound and that damaged by radiant heat suggests that damage produced by ultrasound may be due primarily to the heating action of this form of energy. If necrosis did not occur in tissue which is cooled prior to irradiation with ultrasound, the foregoing suggestion could be accepted without reservation. But necrosis does occur under these conditions even though the maximum temperature recorded during irradiation does not exceed normal body temperature. On the other hand it was found that tissue treated with radiant heat could be protected against damage, by pre-cooling. These facts lead to a possible explanation for the occurrence of necrosis in tissue irradiated with ultrasound. Damage may in fact be due to high temperatures which occur in specific minute regions of liver cells. Acoustic and consequently thermal energy may not be uniformly distributed in cells or tissues because of microscopic inhomogeneities. It was shown

that under certain circumstances damage occurs at the dorsal surface of a liver lobe when the liver is irradiated ventrally with focused ultrasound of one megacycle frequency (fig. 3). The boundary between liver lobes, or between a liver lobe and another tissue apparently constitutes a barrier to the sound beam. Ultrasound reflected from this barrier is absorbed by adjacent tissue. This is one example of a mechanism by which acoustic energy may be concentrated in a local region.

Formed elements and interfaces within cells and between cells may also be responsible for differential absorption of ultrasound. Some minute region of material in the tissue might absorb more acoustic energy than some other minute region of different composition. The temperature rise in the first region would thus be greater than that in the second, and a thermocouple large with respect to either region would give a record of only the average thermal change. Consistent with this possibility is the fact that essentially the same cytological changes occur in livers irradiated with ultrasound as in livers treated with radiant heat. But a thermal change of 35°C . is measured when these changes are produced with radiant heat, as compared to an average thermal change of only 15°C . when ultrasound is used. Thus in livers of animals cooled before irradiation with ultrasound, even though the average thermal change during irradiation does not exceed normal body temperature, local temperatures might. If high local temperatures do occur, they may indeed be a sufficient condition for the occurrence of damage. If, on the other hand, they do not occur, it will be necessary to conclude that damage in pre-cooled livers irradiated with ultrasound is due at least in part, to some non-thermal action of this form of energy.

SUMMARY AND CONCLUSIONS

Livers of mice of strains BUB and BUC were irradiated with focused ultrasound of one megacycle frequency and with unfocused ultrasound of 27 megacycles frequency.

Sinusoids are markedly enlarged and congested immediately following irradiation at either frequency. Many parenchymal cells are vacuolated, and some parenchymal cell nuclei are deformed. The presence of erythrocytes in parenchymal cells and of glycogen granules in sinusoids and small blood vessels is indicative of cell disruption and damage to vascular walls. Disruption of parenchymal cell nuclei was observed only following irradiation at 27 megacycles. Liver tissue in which these changes occur is cut off from the blood circulation immediately, and becomes necrotic within 6 hours.

Tissue necrotized by ultrasound, heat, or ligation autolyses more slowly than tissue necrotized by ordinary chemical or surgical means. The slow rate of lysis is attributed to vascular occlusion.

Following injury to the liver with ultrasound of one or 27 megacycles frequency, mitosis does not begin within three days as it does after chemical or surgical damage. Mitotic activity is delayed until the 5th day after injury. Cauterization of part of a liver lobe also results in a delay of mitotic activity. Cell divisions are not apparent until the 4th day after injury. The fact that the delay occurs supports the hypothesis that the onset of mitosis depends upon the course of events in the zone of injury.

During 15 seconds of irradiation with focused ultrasound of one megacycle frequency (40 watts delivered to the lens) the temperature rises in the liver at the rate of 1°C./sec. However the occurrence of liver necrosis is not prevented by cooling animals to 10°C. before exposing them to sound for 15 seconds. Necrosis is prevented, on the other hand, by pre-cooling, when the same rate of change of temperature occurs in livers treated for 15 seconds with concentrated radiant heat. It is concluded that the average thermal change is not responsible for damage produced by ultrasound in pre-cooled livers.

Cytological modifications which occur in livers treated with radiant heat are essentially like those observed in livers irradiated with ultrasound. The respective modifications are

most alike, however, when the thermal change measured in heat treated livers is more than twice as great as that measured in livers irradiated with ultrasound. It is suggested that damage in tissue irradiated with ultrasound may be due to high local temperatures which the thermocouple does not "see."

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MEMBRANE POTENTIAL CHANGES AND ION MOVEMENTS IN THE FROG SARTORIUS MUSCLE

WILLIAM K. STEPHENSON

Department of Zoology, University of Minnesota, Minneapolis, Minnesota

FOUR FIGURES

Muscle cells maintain a high potassium concentration and exclude sodium and chloride against strong chemical diffusion gradients (see table 1). Concomitant with this ion distribution there exists a resting membrane potential of 80 to 100 mV., the fiber interior being negative with respect to the medium.

TABLE 1
Ion composition of frog muscle and blood

| | mEq./LITER WATER IN | | |
|-----------|---------------------|---------------------|--------------------|
| | Fibers ¹ | Fibers ² | Blood ³ |
| Sodium | 16.5 | 17.0 | 108 |
| Potassium | 127 | 124 | 2.6 |
| Chloride | | 1.3 | 77 |

¹ Values determined in the course of this study. Each figure represents an average of 8 independent determinations.

² Calculated from the data of Fenn ('36). In all calculations the water content of frog muscle used was 80% of the fresh weight, that of the plasma was 96% (Fenn, '36), and the extracellular space volume used was 13.0% (Boyle, Conway, Kane and O'Reilly, '41).

There are two general membrane mechanisms (disregarding hydrodynamic considerations) which could account for the high concentration of potassium within the muscle fibers (see Teorell, '49). One is the accumulation of potassium by means of an inwardly directed secretory mechanism. The other is the electrical membrane potential which, if established

³ Present address: Department of Biology, Earlham College, Richmond, Indiana

by a mechanism independent of outward potassium diffusion, could cause an accumulation of potassium in response to the electrical gradient across the fiber membrane. The extrusion of sodium against a net electrochemical gradient has been noted for a number of tissues and has been suggested as a possible source of membrane potential. Experimental evidence justifying this concept is summarized by Hodgkin ('51).

If either of the above two mechanisms is operating the relationship between the membrane potential and potassium distribution at steady state conditions can be approximated by the Nernst equation:

$$E = \frac{R}{n} \frac{T}{F} \log_e \frac{f_i C_{K_i}}{f_o C_{K_o}} \quad (1)$$

where E = the membrane potential, R = the gas content, T = the absolute temperature, n = the charge carried by each ion, F = the Faraday, f_i = the activity coefficient for the potassium ion inside the fibers, f_o = the activity coefficient for the potassium ion in the bathing medium, C_{K_i} = the concentration of potassium within the fibers, and C_{K_o} = the concentration of potassium in the medium. The assumption is usually made that f_i and f_o are equal in the intracellular and extracellular phases. This assumption would not be valid in a system in which potassium is accumulated by adsorptive binding to molecules confined within the cell.

This equation is equally applicable to any other ion in muscle whose movement across the fiber membrane is not restricted by an energy utilizing process, i.e. any ion which is passively distributed.

Some insight as to the relative contributions of sodium extrusion and potassium accumulation to the membrane potential might be gained if experimental conditions could be realized under which the sodium extrusion mechanism could be suddenly activated and the time course of subsequent membrane potential changes followed. If sodium is extruded as an ion, its extrusion should bring about a sudden increase in membrane potential. This increase in the electrical gradient would cause the passive accumulation of potassium limited by the permeability of the membrane to the influx of that ion. Prior to the attainment of steady state conditions the

electrical membrane potential should be greater than the chemical diffusion potential for potassium

$$\left(> \frac{RT}{nF} \log_e \frac{K_i}{K_o}\right).$$

On the other hand, if potassium is accumulated by an active transport mechanism (presumably electroneutral) the membrane potential would arise from the outflux of potassium in response to its chemical gradient. In this case equation (1) should hold throughout potassium accumulation and sodium extrusion.

The conditions necessary for this experiment exist in the work of Steinbach ('40, '51, '52). If muscles are soaked in cold potassium-free saline for 24 hours they lose potassium and gain sodium. Upon transfer to high-potassium saline (10 mEq./l.) at room temperature for one hour, the muscles extrude sodium and reaccumulate potassium in approximately equi-equivalent amounts of about 15 mEq./Kg of muscle.

The work reported in this paper is an attempt to follow the membrane potential over an interval during which such rapid ion shifts are taking place.

METHODS

Frogs used in these studies were commercially supplied *Rana pipiens*, which were stored at about 20°C. prior to use. Table 2 lists the composition of the experimental solutions.

Preparatory to sodium and potassium analyses, a muscle was removed from saline solution, trimmed of its tendonous ends, blotted quickly on clean filter paper, and weighed to the nearest milligram on a 500 mg torsion balance. The muscles were then placed in 15 ml graduated centrifuge tubes, one-half ml of concentrated nitric acid was added, and digestion was accomplished by heating for one hour over a boiling water bath. After cooling, the preparations were diluted to 15 ml. The actual analyses were carried out with a Perkin and Elmer flame photometer using the direct com-

TABLE 2
Experimental solutions

| | POTASSIUM-FREE SALINE SOAKING SOLUTION | HIGH-POTASSIUM SALINE RECOVERY SOLUTION |
|--|---|--|
| | <i>mM/l</i> | <i>mM/l</i> |
| NaCl | 120 | 110 |
| KCl | none | 10.0 |
| (Na) ₂ PO ₄ buffer | 5.0 | 5.0 |
| pH | 7.2 | 7.2 |

All solutions were made up in water which had been: (1) distilled in a copper-lined still, and (2) either redistilled in a glass still or passed through "Deeminite" ion exchange resin.

parison method. Standards contained 3.33% nitric acid by volume.

Muscle chlorides were determined using the gravimetric technique outlined by Williams, Seibert, and Huggins ('54). This procedure does not suffer from the errors involved when the Vollhard titration for chloride is used for biological material (see Shenk, '50; Williams, Seibert, and Huggins, '54). Ten muscles were pooled for each analysis.

In calculating the fiber ion concentrations for experimental muscles the same constants were used as for fresh muscle (table 1). An average water content of 80.7% was found for 4 sartorii which had been soaked for 24 hours in potassium-free saline at 2-6°C. Desmedt ('53) determined an average extrafiber space value of 12.5% for muscles which had been soaked for extended periods in potassium-free Ringer. Thus the figures used should not introduce serious error in the calculations.

Membrane potential measurements were made utilizing the microelectrode technique of Ling and Gerard ('49a). The microelectrodes used had a tip diameter of less than one micron and were filled with 3 M. KCl.

During electrical measurements the muscles were placed in an 80 ml capacity chamber constructed of plastic and paraffin wax. Its design permitted aeration and circulation of the saline bathing the muscle. The muscles were stretched to

about body length and pinned with steel needles under slight tension while in the muscle chamber.

An indifferent electrode consisting of a 3 M. KCl-agar bridge also dipped into the solution bathing the muscle in the chamber. Both the microelectrode and the indifferent electrode were connected to the recording apparatus thru calomel half-cells and beakers of 3 M. KCl or directly through chlorided silver electrodes.

The amplifier circuit consisted of a high-input-resistance, direct current amplifier and oscilloscope. Membrane potential values were recorded as vertical deflections on the oscilloscope screen.

Calculations of standard error of the mean and correlation coefficients were carried out in accord with methods outlined in Snedecor ('46).

Experimental procedures

The general experimental plan of treating muscles was modified from Steinbach ('40, '51). For the work involving membrane potential measurements longitudinally split sartorius muscles were used. Each half was immersed in 25 ml of potassium-free saline and placed in the ice box at 2–6°C. for 11 to 18 hours. During this soaking period in the cold, potassium leaves the muscle fibers in exchange for sodium. Following soaking, one half of each muscle was carefully blotted, trimmed, weighed, and transferred to a 15 ml centrifuge tube for digestion. The other half was transferred to 10 mM. KCl saline in the muscle chamber for 50 minutes during which time membrane potential measurements were made. This period of exposure to 10 mM. KCl saline will be referred to as the recovery period since the muscles reaccumulate potassium and reeliminate sodium. In every case tested muscles undergoing the above treatments contracted when stimulated by mild electric shock.

The first set of potential determinations was initiated as soon as possible after transfer of the muscle to the muscle

chamber. These determinations were completed within 10 minutes, and the muscle remained in the chamber for 30 additional minutes before the second set of potential measurements was started. The latter also consumed 10 minutes time, so that the entire recovery period in high-potassium saline covered 50 minutes.

Both sets of membrane potential determinations were made on the same 10 muscle fibers. The electrode punctures at the end of recovery were made at a distance of several hundred micra from the punctures made at the beginning of recovery. This procedure should not result in a decreased second reading due to fiber damage since Nastuk and Hodgkin ('50) have shown that the insertion of a second microelectrode 200 μ from a recording microelectrode causes a potential decrease of less than 1-2%.

After completion of the second potential readings, the recovered muscles were also trimmed, blotted, weighed, and transferred to 15 ml centrifuge tubes for digestion and cation analysis.

Chloride concentrations within the fibers of soaked and recovered muscles were determined from pooled groups of 10 whole sartorii each. Paired muscles from 10 frogs were soaked for 18 hours in potassium-free saline at 2-6°C. At the end of this time, one member of each pair was weighed and placed in a common 15 ml centrifuge tube for digestion. The other member of each pair was transferred to 10 mM. KCl saline at room temperature for 50 minutes. After recovery these muscles were also weighed and placed in a centrifuge tube for digestion.

RESULTS

Soaking for 11 to 18 hours in potassium-free saline in the cold resulted in a loss of fiber potassium from the normal condition in table 1 to an average of 65.7 ± 3.3 mEq./liter fiber water (lfw.) and a gain of fiber sodium to 101.5 ± 4.6 mEq./lfw. Soaked muscle halves which were transferred to 10 mM. KCl saline for 50 minutes at room temperature ac-

cumulated potassium to a fiber concentration of 96.4 ± 3.5 mEq./lfw. and extruded sodium to a concentration of 70.5 ± 4.0 mEq./lfw. Thus, during recovery in 10 mM. KCl saline the muscles gained an average of 30.7 ± 2.9 mEq./lfw. of potassium and lost an average of 30.9 ± 2.7 mEq./lfw. of sodium.

Following 18 hours of soaking in potassium-free saline the fiber concentration of chloride had risen to 56 mEq./lfw. Transfer to 10 mM. KCl saline for one hour resulted in only a slight decrease in fiber chloride to 53 mEq./lfw.

The average membrane potential recorded within the first 10 minutes after transfer to 10 mM. KCl saline at room temperature was 40.1 ± 1.1 mV., and that recorded 40 to 50 minutes after transfer was 40.0 ± 1.0 mV. Thus during an interval in which rapid shifts of sodium and potassium were taking place the average membrane potential change was only -0.1 ± 0.9 mV.

The foregoing results are summarized in table 3.

Ion diffusion gradients as sources of membrane potential

Since potassium and chloride ions are able to permeate the fiber membrane (Levi and Ussing, '48; Harris and Burn, '49), the diffusion of each of these ions in response to its existing concentration gradient could produce an electrical potential in accord with equation (1). From this equation and the average ion concentrations of the muscle fibers from table 3 and of the bathing media from table 2, it is possible to calculate the maximum size of the electrical potential which could result from the diffusion of each ion both at the beginning and end of recovery.

For potassium the diffusion gradient could give rise to a membrane potential of about 48 mV. at the outset of recovery and 57 mV. at the end of recovery. The diffusion of chloride could only support an electrical potential of about 19 mV. at the beginning of recovery and 21 mV. at the end. Sodium would support an electrical potential of the opposite sign than

TABLE 3
Potassium and sodium concentrations and membrane potentials of experimental muscles

| MUSCLE NUMBER | SOAKING TIME | mEq/L FIBER WATER | | | | MEMBRANE POTENTIAL IN mV | | | |
|------------------|-----------------|-------------------|----------------|-----------------|-----------------|--------------------------|-----------------|---------------|------------------------------|
| | | K ₁ | K ₂ | K change | Na ₁ | Na ₂ | Na change | Pot. 1 | Pot. 2 Pot. change |
| 1 | 16 | 67 | 112 | + 45 | 101 | 60 | - 41 | 37.6 | 37.7 + 0.1 |
| 2 | 15 | 78 | 97 | + 19 | 75 | 46 | - 29 | 35.8 | 38.8 + 3.0 |
| 3 | 12 | 84 | 104 | + 20 | 70 | 55 | - 15 | 29.8 | 32.4 + 2.6 |
| 4 | 11 | 84 | 111 | + 27 | 77 | 52 | - 25 | 15.5 | 18.4 + 2.9 |
| 5 | 11 | 64 | 112 | + 48 | 105 | 74 | - 31 | 44.0 | 43.5 - 0.5 |
| 6 | 11 | 65 | 81 | + 16 | 99 | 84 | - 15 | 53.7 | 48.1 - 5.6 |
| 7 | 12 | 46 | 92 | + 46 | 132 | 84 | - 48 | 35.6 | 42.0 + 6.4 |
| 8 | 12 | 55 | 91 | + 36 | 108 | 63 | - 45 | 44.8 | 40.0 - 4.8 |
| 9 | 12 | 44 | 74 | + 30 | 121 | 98 | - 23 | 41.0 | 39.8 - 1.2 |
| 10 | 16 | 70 | 106 | + 36 | 105 | 65 | - 40 | 42.3 | 38.3 - 4.0 |
| 11 | 17 | 82 | 117 | + 35 | 86 | 52 | - 34 | 45.0 | 34.6 - 10.4 |
| 12 | 18 | 58 | 96 | + 38 | 108 | 82 | - 26 | 31.8 | 38.9 + 7.1 |
| 13 | 15 | 60 | 79 | + 19 | 104 | 85 | - 19 | 47.4 | 46.7 - 0.7 |
| 14 | 16 | 72 | 87 | + 15 | 112 | 79 | - 33 | 50.8 | 49.0 - 1.8 |
| 15 | 17 | 57 | 87 | + 30 | 119 | 79 | - 40 | 45.7 | 51.5 + 5.8 |
| Averages | | 65.7 ± 3.3 | 96.4 ± 3.5 | + 30.7 ± 2.9 | 101.5 ± 4.6 | 70.5 ± 4.0 | - 30.9 ± 2.7 | 40.1 ± 1.1 | 40.0 ± 1.0 - 0.1 ± 0.9 |

Sartorius halves were soaked for 11 to 18 hours in K-free saline at 2-6°C. One member of each muscle half pair was recovered in 10 mM KCl saline for 50 minutes. K₁ = fiber potassium concentration prior to recovery. K₂ = fiber potassium concentration at the end of recovery. K change = K₂ - K₁ = potassium accumulated by the fibers during recovery. Na₁ = fiber sodium concentration prior to recovery. Na₂ = fiber sodium concentration at the end of recovery. Na change = Na₂ - Na₁ = sodium extruded by the fibers during recovery. Pot. 1 = average membrane potential recorded from 10 fibers at the beginning of recovery. Pot. 2 = average membrane potential recorded from 10 fibers near the end of recovery. Pot. change = Pot. 2 - Pot. 1 = average membrane potential change during recovery. Fiber concentrations of sodium and potassium were calculated using the constants noted in table 1. ± values are standard errors.

that observed. Thus, of the three most abundant monovalent ions in the system, only potassium is distributed such that its diffusion could give rise to the membrane potential observed at the onset and close of the 50 minute recovery period.

However, three lines of reasoning lead to the conclusion that the observed membrane potential is not primarily a potassium diffusion potential:

(a) The fiber concentration of potassium was observed to increase from 66 to 96 mEq./lfw. during recovery. If the membrane potential is primarily a potassium diffusion potential, it should also show an increase corresponding to the increase in the potassium diffusion gradient. From equation (1) this increase is calculated to be 9 mV., yet the observed change was zero. Either the membrane potential is not a potassium diffusion potential or the failure of the membrane potential to increase is the result of short circuiting effects caused by an increased permeability of the membrane to other ions.

(b) If the observed membrane potential is a potassium diffusion potential there should be a correlation between the change in fiber potassium during recovery and the change in membrane potential for each individual muscle. (Individual muscles varied rather widely in each class of measurements.) This should hold even if the increase in short circuiting is sufficient to constrain a potassium diffusion potential to a constant level during the recovery period. In other words, those muscles which reaccumulated the greatest amount of potassium should show a slight potential increase, those reaccumulating an average (ca. 30 mEq.) amount should show no change, and those which reaccumulated the least potassium should show a decrease in membrane potential.

A test of this hypothesis has been prepared from the data of table 3 and is shown in table 4, number 1. Potassium accumulation and membrane potential change are not significantly correlated. This further indicates that the maintenance of the membrane potential in the experimental muscles is independent of the outward diffusion of potassium.

TABLE 4

Correlations between experimental variables

| RELATIONSHIP TESTED | | CORRELATION COEFFICIENT | LEVEL OF SIGNIFICANCE |
|--|---|----------------------------------|--------------------------|
| 1. Membrane potential change during recovery | <i>vs.</i> Potassium accumulation during recovery | + 0.119 | 67 |
| 2. Membrane potential change during recovery | <i>vs.</i> Sodium extruded during recovery | + 0.066 | 84 |
| 3. Membrane potential at the beginning of recovery | <i>vs.</i> Fiber potassium conc. before recovery | (lin.) — 0.340 (log.) — 0.330 | 22 23 |
| 4. Membrane potential at the end of recovery | <i>vs.</i> Fiber potassium at the end of recovery | (lin.) — 0.597 (log.) — 0.592 | 02 02 |
| 5. Membrane potential at the beginning of recovery | <i>vs.</i> Sodium extruded during recovery | + 0.091 | 74 |
| 6. Membrane potential at the end of recovery | <i>vs.</i> Sodium extruded during recovery | + 0.274 | 32 |

(c) Further, a straight line (or nearly so) should result when membrane potential is plotted against the log of the fiber potassium concentration, in accord with equation (1). Small deviations from linearity could result from electrical short circuiting due to the leakage of sodium, chloride, or other ions through the fiber membrane in response to their respective electrochemical potential gradients.

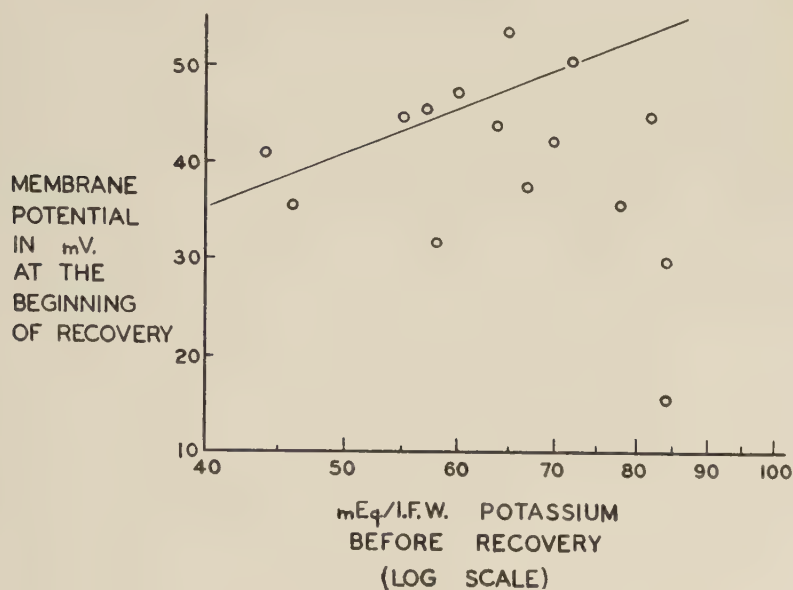


Fig. 1 The relationship between membrane potential at the beginning of recovery and log fiber potassium before recovery. Data was taken from table 3.

If the fiber potassium concentration prior to recovery is plotted against the membrane potential observed at the beginning of recovery, the data result in the scatter of points shown in figure 1 (see also number 3 of table 4). The straight line in figure 1 represents the theoretical potential calculated from equation (1) and neglects short circuiting effects. At the outset of recovery the positive correlation between potassium distribution and membrane potential predicted by equation (1) does not exist. This observation was also made by Tobias

(50) who found no such correlation in muscles which had been soaked in potassium-free saline at 4–6°C. for up to 100 hours.

In the above comparison potential measurements and ion analyses were made on different members of each split-half pair. This difficulty is not encountered in plotting the fiber potassium against membrane potential at the end of the re-

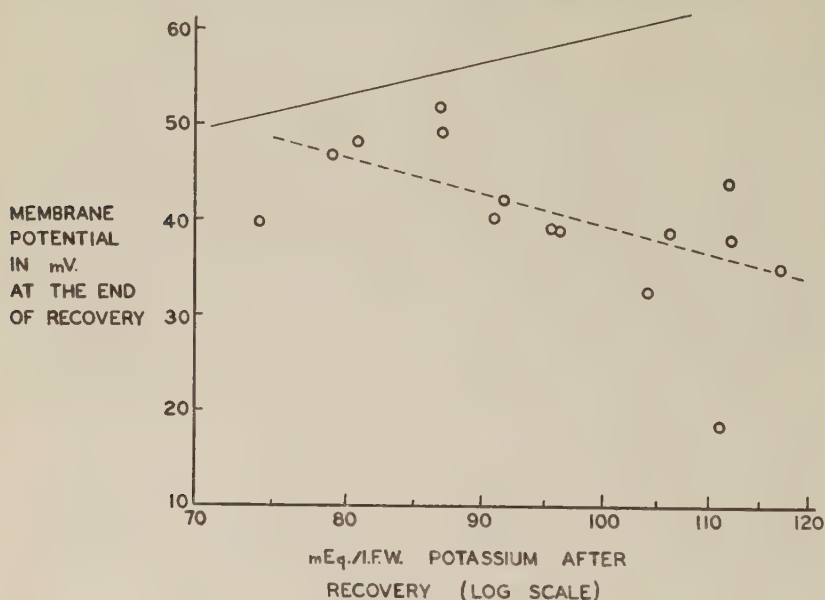


Fig. 2 The relationship between membrane potential at the end of recovery and log fiber potassium after recovery. Data was taken from table 3.

covery period, for both ion analyses and potential determinations were carried out on the same sartorius muscle half. This plot is shown in figure 2 (see also number 4 of table 4). Here the departure of the points from the theoretical line drawn from equation (1) is much clearer than in figure 1. The experimental points suggest a line which has a negative slope, while the theoretical line has a positive slope. This is clear cut evidence that the membrane potential measured at the close of the recovery period is not primarily a potassium diffusion potential.

Evidence for the active accumulation of potassium

Equation (1) relates the potassium distribution and membrane potential under steady state conditions. The muscles which have undergone experimental treatment in this study are not at steady state, for potassium is accumulated rapidly throughout the recovery period. If potassium moves passively in response to an increased electrical potential, the membrane potential should be greater than the chemical diffusion potential throughout the recovery period

$$(E > \frac{RT}{nF} \log_e \frac{C_{K1}}{C_{K0}}).$$

This condition is not met or even closely approached at any time during recovery. The membrane potential, in fact, remains constant at 40 mV. while the fiber potassium rises to a mean concentration which would require a potential of at least 57 mV. to bring about its passive accumulation. Potassium has thus been moved against a net electrochemical gradient.

Further, if potassium is passively accumulated the membrane potential recorded at the end of recovery should be positively correlated with the fiber potassium (i.e. those muscles with the greatest average membrane potentials should have the greatest fiber potassium concentration). This expected positive correlation should follow the solid line in figure 2 if the system is at or very near to steady state. It might depart from the line, though roughly parallel it, if the system is approaching steady state from some distance. The observed correlation is negative as has been previously noted. This also indicates that potassium does not move passively during recovery but is actively accumulated.

Sodium extrusion as a possible source of membrane potential

As outlined in the introduction, the ionic extrusion of sodium could be directly responsible for maintaining the membrane

potential. If this is so, the individual sartorii which extrude the greatest quantity of sodium ions would be expected to show the greatest potential increase during recovery. Membrane potential change during the course of recovery is compared with sodium extruded in table 4, number 2. There is no significant correlation between the two factors.

Steinbach ('52) and Desmedt ('53) have studied the time course of sodium extrusion and report a half time of about 30 minutes, with sodium extruded most rapidly at the beginning of recovery. If extrusion is accomplished by an ionic sodium pump, the membrane potential should rise rapidly following its activation. In those muscles extruding the greatest amount of sodium the rate of extrusion should be most rapid, and hence the membrane potential should be greatest at the beginning of recovery. The size of the average membrane potentials of individual muscles should, then, be positively correlated with the amount of sodium extruded. Figure 3 and ranks 5 and 6 of table 4 illustrate data which test this relationship both at the beginning and end of recovery. It is apparent that the expected correlation is not significant.

Neither the potential change during recovery nor the absolute magnitude of the membrane potential is well correlated with the amount of sodium extruded. Thus the extrusion of sodium is not directly responsible for the observed electrical potential.

*A membrane potential generator which is independent
of potassium, sodium and chloride distributions*

Previous sections of this paper have demonstrated that the observed membrane potentials in muscles which have undergone experimental treatment are not dependent upon existing potassium, sodium, and chloride distributions or the movements of these ions. These results lead to the postulation of a mechanism independent of the three ions which is capable of establishing and maintaining the membrane potential at a level of about 40 mV. The results of these studies afford

further positive evidence for the existence and properties of such a membrane potential generator.

In figure 3 the individual potential measurements made at the beginning and end of the recovery period are connected by arrows indicating the direction of potential change. In 14 of the 15 pairs of measurements the potential changed toward the mean value of 40 mV. In one case the potential did not move toward 40 mV., and in another the potential change was so large that the final potential overshoot the 40 mV. value by over 2 mV.

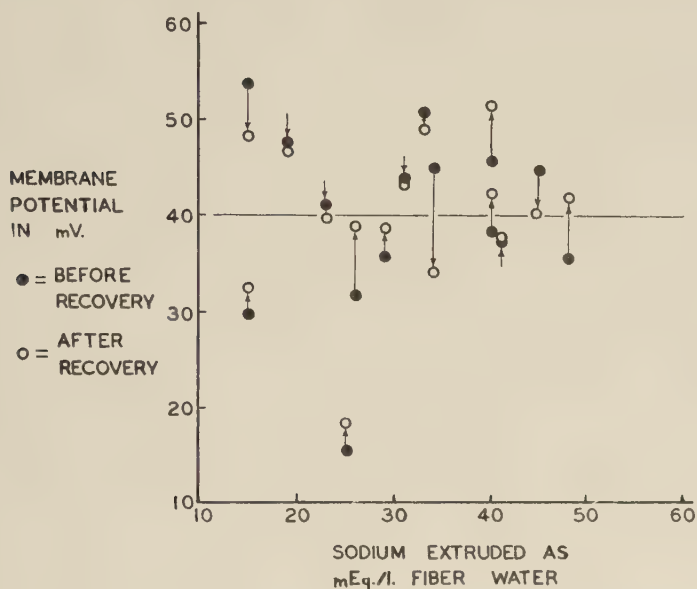


Fig. 3 The direction of membrane potential change during recovery plotted against sodium extruded during recovery. Corresponding individual potential values are connected by arrows indicating the direction of change during recovery.

Since the average potential change during the recovery period is nearly zero (0.1 ± 0.9 mV.) it would be expected, a priori, that the membrane potential change of each individual muscle half would be random. That is, the potential of each muscle would be expected to have a probability of $\frac{1}{2}$ of increasing and $\frac{1}{2}$ of decreasing during recovery. The probability of 14 potentials out of a sample of 15 changing

toward the mean during recovery is less than one in two thousand. In a similar independent set of experiments the membrane potential regulated toward the mean in 9 of 12 sets of measurements. The probability in this case is ca. $1/14$ that 9 or more of the potentials should so change. The mean potential at the beginning of recovery for these data was 43.9 ± 1.6 mV. for 10 individual fiber measurements on 12 sartorii.² These determinations were made on summer frogs while the original data were collected from winter frogs, which may account for the difference in the mean potentials. The product of the two above probabilities is ca. $1/28,000$ that 23 or more individual potentials out of 27 should change toward the mean.

Since this regulatory tendency exists, those potential values which depart furthest from 40 mV. at the beginning of recovery would be expected to show the greatest change during recovery. In figure 4 the results from table 3 and those of the independent set of experiments have been superimposed to test this relationship. The two sets of points in figure 4 have been plotted on separate abscissas so that the means of 40 and 44 mV. are superimposed. This was done because the tendency of the potential change during recovery is toward the mean in each case. From this plot it can be seen that the relationship between initial potential and potential change is negative and probably non-linear.

DISCUSSION

The working hypotheses for this study outlined in the introduction are based upon the assumption that equation (1) is applicable to relate potassium distribution and membrane potential in excitable tissue (Hodgkin, '51, and others). The foregoing results, however, demonstrate the inadequacy of this assumption under the experimental conditions employed. A similar conclusion has been reached in studies involving the microinjection of KCl into squid axons (Grund-

² Only nine determinations were made for two of the sartorii in these measurements.

fest, Kao, and Altamirano, '54) and frog muscle (Falk and Gerard, '54). Here the internal potassium concentration, and consequently the K_i/K_o ratio, can be increased without an appreciable effect on the electrical membrane potential. Using reversible Ag-AgCl electrodes Mauro ('54) recorded a chloride potential of ca. 35 mV. which indicates that chloride is not passively distributed in accord with equation (1). Thus the application of the Nernst equation to biological systems is now open to serious question.

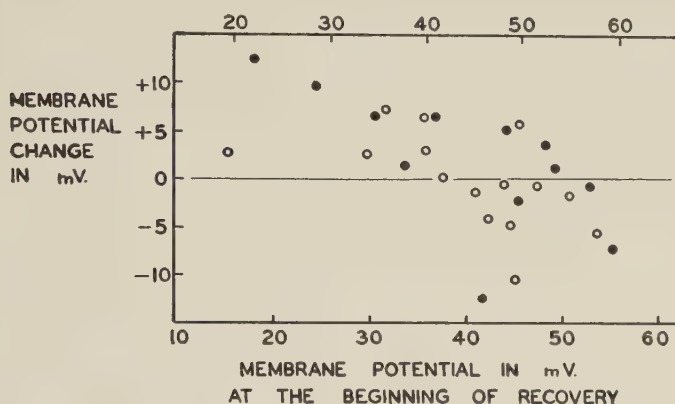


Fig. 4 The relationship between membrane potential change during recovery and the membrane potential at the beginning of recovery. Data from an independent experiment (solid circles, see text) has been superimposed on data from table 3 (open circles). The abscissa at the top of the graph is for the superimposed data and that at the bottom is for the data of table 3.

In the research reported it has been demonstrated that the membrane potential did not change while rapid movements of sodium and potassium occurred. Shaw and Simon ('55) have also found that the membrane potential did not change while sodium was being extruded in toad muscle. With squid axons, Hodgkin and Keynes ('55) have shown that dinitrophenol and cyanide decrease sodium extrusion and potassium accumulation without effect on the membrane potential. Desmedt ('53) has carried out some investigations of this type, but in only one case was his recovery period bracketed by

membrane potential measurements made on muscle in media of the same potassium concentration. In this instance he observed an increase in membrane potential concomitant with an increase in fiber potassium. Desmedt's soaking and recovery solutions were modifications of Ringer which contained calcium, while my experimental solutions did not. Since calcium has been shown to decrease the permeability of many tissues to salts (True, '14; Aspery, '33; Wilbrandt, '40; Stephenson, unpublished), it is possible that the omission of calcium from the solutions used in this study may explain the variance with Desmedt's results.

An examination of the relationship between membrane potential and sodium extrusion reveals that there is no significant correlation between (a) the amount of sodium extruded and the membrane potential change during recovery and (b) the magnitude of the membrane potential and the fiber sodium concentration either at the beginning or end of recovery. These observations indicate that sodium was not primarily extruded as an independent ion, but in close one for one combination with the movement of some other ion across the fiber membrane (either the inward secretion of a positive ion or the outward secretion of a negative ion). Since chloride does not shift appreciably during recovery,² and potassium moves into the fibers in approximately an equi-equivalent amount to the sodium extruded, it seems likely that the coupling exists between sodium and potassium movements. This conclusion is supported by the studies of Hodgkin and Keynes ('53 and '55) with *Sepia* axons and Keynes ('54) with frog muscle. They found that the removal of potassium from the external medium reversibly decreased the sodium outflux, indicating a coupling between sodium extrusion and potassium influx. Such a coupling is also consistent with the work of Steinbach ('52) on frog *sartorii* which demonstrated the dependence of potassium accumula-

² Steinbach ('40) reports a slight increase of muscle chloride during recovery, while the results of this study indicate a slight decrease in fiber chloride.

tion upon sodium extrusion under conditions which were calculated to be near optimal for the former process.

It is not, however, consistent with the results of Shaw and Simon ('55) obtained from toad muscle. They utilized experimental conditions whereby sodium was extruded without an increase in muscle potassium or a change in membrane potential. Although they did not observe reciprocal sodium and potassium movements, their sodium extrusion mechanism is still an electroneutral process since membrane potential did not change during its activity. This implies that another ion moves in exchange for or combination with sodium. It should also be noted that Hodgkin and Keynes's coupling was not complete, for some sodium extrusion took place even in potassium free solutions.

The existence of a mechanism for maintaining a membrane potential which is independent of sodium and potassium movements is not an entirely new concept. Tobias's work ('50) has previously demonstrated the existence of such a mechanism in muscles soaked for extended periods in distilled water in the cold. He found that the resulting non-excitables, water logged muscles maintained a membrane potential of 38 mV. even though 99% of their potassium and 93% of their sodium had been leached out. His discussion concerning speculation as to the possible sources of such a potential is germane to the results of this study. Grundfest ('55) has summarized other evidence which indicates that it is not possible to explain the membrane potential exclusively in terms of sodium, potassium and chloride distributions and movements.

Ling and Gerard ('49b) have divided the resting potential into two components: an A potential between 55 to ca. 85 mV. whose magnitude is correlated with the amount of high energy phosphate in the muscle fiber, and a B potential between 0 to 55 mV. which is independent of high energy phosphate and demonstrable after iodoacetate poisoning and tetanus. It seems reasonable to suggest that the distilled water soaked potential, B potential, and the K-Na-Cl independent potential

of this study may arise from the same mechanism since they are of approximately the same magnitude and are all observed under conditions of stress (poisoning and tetanus or prolonged cold soaking).

One of the significant features of this study is that such a potential is demonstrable in muscles which have undergone much less rigorous experimental treatment than in the other experiments. Tobias's muscles were leached of virtually all their potassium and were inexcitable. Ling and Gerard's muscles were poisoned and tetanized. By contrast, the fibers of the muscles used in this report retained from $\frac{1}{2}$ to $\frac{2}{3}$ of their potassium and had 3 to 5 times their normal sodium concentration. In addition, they were excitable by directly applied electric shock and were able to extrude large quantities of sodium and reaccumulate large amounts of potassium.

The contribution which this K-Na-Cl independent component of the resting potential makes to "normal" resting potential is not at all clear. In order to make its presence felt in fresh muscle the generator would have to be capable of supporting membrane potentials considerably in excess of the 40 mV. observed. This is particularly true since the outward diffusion of potassium can theoretically maintain a much higher voltage (in excess of 90 mV.).

ACKNOWLEDGMENTS

I would like to express particular gratitude to Dr. H. Burr Steinbach whose guidance and counsel have been most helpful in the execution of this work. Dr. Otto Schmitt, John Trank, and Kent Chapman have given assistance with some aspects of the electronics involved, and Dr. H. Burr Steinbach, Dr. Otto Schmitt, Dr. William Battin, Dr. John Davison, and Jane Stephenson have read and criticized various portions of the paper. Parts of this work were supported by a Fellowship from the Greater University Fund, University of Minnesota, Minneapolis, Minnesota and by Contract Nonr-24900 Office of Naval Research administered by Dr. H. B. Steinbach.

ADDENDUM

For other recent information on the relationship between cellular potassium concentrations and membrane potentials consult: Ardian, R. H. 1956. The effect of internal and external potassium concentrations on the membrane potential of frog muscle. *J. Physiol.*, 133: 631-658; Altamirano, M. and C. W. Coates 1957. Effect of potassium on electroplax of *Electrophorus electricus*. *J. Cell. Comp. Physiol.*, 49: 69-101; Shaw, F. H., Shirley E. Simon, and B. M. Johnstone 1956. The non-correlation of bioelectric potentials with ionic gradients. *J. Gen. Physiol.*, 40: 1-17.

SUMMARY

1. Frog sartorius muscles were soaked in potassium-free saline for 11 to 18 hours at 4-6°C. Their fiber potassium concentrations dropped to about $\frac{1}{2}$ to $\frac{2}{3}$ of normal and their fiber sodium increased 3-5 times. Following transfer to 10 mM. KCl saline at room temperature (25°C.), the fibers accumulated an average of 31 mEq./l. fiber water potassium and extruded 31 mEq./l. fiber water sodium within 50 minutes (recovery period).

2. Membrane potentials were recorded at the beginning and the end of the recovery period with the glass microelectrode technique. Throughout recovery the average membrane potential remained constant at 40 mV.

3. The 40 mV. membrane potential observed is not primarily a potassium diffusion potential as evidenced by the following:

- (a) The membrane potential remained unchanged while fiber potassium increased 50% during recovery.
- (b) For individual muscles there was no correlation between the potential changes during recovery and the amounts of potassium accumulated.
- (c) Log fiber potassium plotted against membrane potential did not give a straight line relationship in accord with the Nernst equation.

4. The accumulation of potassium during recovery is an energy utilizing process. This conclusion is supported by the observations that:

- (a) The electrical membrane potential was not large enough at any time during recovery to cause the net passive influx of potassium.
- (b) Membrane potentials at the end of recovery plotted against log fiber potassium concentrations result in a line which has a negative slope, rather than a positive slope as predicted by the Nernst equation.

5. The extrusion of sodium ions is not directly responsible for the observed membrane potential since:

- (a) The potential changes during recovery were not correlated with the amounts of sodium extruded.
- (b) The absolute magnitudes of the membrane potential were not correlated with the amounts of sodium extruded.

6. The results are taken to indicate that sodium extrusion and potassium accumulation are directly coupled, so that these ions move in a one for one electroneutral exchange during recovery.

7. The membrane potential has a marked tendency to change toward 40 mV. during recovery. The magnitude of the change is greatest for those muscles whose average potential is furthest from 40 mV. at the beginning of recovery.

8. The observed membrane potential of 40 mV. is maintained by a mechanism which is independent of the distributions or movements of either potassium, sodium, or chloride ions.

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EXCRETION OF NITROGEN BY THE ALLIGATOR EMBRYO ¹

HUGH CLARK, BETTY SISKEN AND JOHN E. SHANNON

Department of Zoology, University of Connecticut

TWO FIGURES

Embryos of the black snake were found (Clark, '53) to excrete 60% of their nitrogenous waste as urea, 20% as uric acid and 10% as ammonia. Since this performance was in sharp contrast with the expectations as summarized by Needham ('31, '50) the investigation of nitrogen partition in the excreta of another reptilian group was undertaken.

Eggs of *Alligator mississippiensis* were shipped to our laboratory via air express from Avery Island, Louisiana.² Late embryos were incubated at 90°F. in damp sphagnum moss. For analysis of the excreta, embryos were divided into the fractions: embryo, yolk plus yolk sac, allantoic fluid and white. Total excreted nitrogen is calculated as the sum of the fractions, except in the last two embryos of which homogenates of the entire egg content were made. Homogenization was accomplished in a Sorvall Omnimixer. Methods of nitrogen analysis have been described previously (Clark, '53).

RESULTS

Because of difficulty in incubating the earliest stages, the growth data presented in table 1 show the growth rate under natural conditions. The youngest embryos are three weeks of age (observation of time of laying by Mr. Simmons) and are

¹ This study was supported by Grant G-3827(c) from the U.S. Public Health Service of the National Institutes of Health.

² We are indebted to Mr. E. M. Simmons of the E. A. McIlhenny Estates, Avery Island, Louisiana for several shipments of eggs.

TABLE 1

Distribution of excreted nitrogen among components of the alligator egg, mg N

| | 0 | AGE, DAYS | | | | | | | | | | 49 | 54 |
|---------------------------------------|---------|-----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|----|----|
| | | 20 | 24 | 27 | 32 | 36 | 36 | 43 | 47 | 49 | 54 | | |
| Embryo wet wt., gm | Unfert. | 1.71 | 2.39 | 2.81 | 3.21 | 17.30 | 18.03 | 22.75 | 34.60 | 35.26 | 48.52 | | |
| Ammonia | | | | | | | | | | | | | |
| Embryo | ... | 0.07 | 0.09 | 0.13 | 0.13 | 1.08 | 0.97 | 1.90 | 1.56 | | | | |
| Yolk and yolk sac | 0.45 | 0.64 | 0.66 | 0.93 | 0.98 | 0.94 | 0.92 | 1.00 | 1.39 | | | | |
| White | 0.05 | ... | 0.10 | 0.15 | 0.48 | ... | ... | ... | | | | | |
| Allantoic fluid | ... | ... | 0.03 | 0.18 | 0.02 | 0.07 | 0.19 | 0.60 | 3.10 | | | | |
| Total NH_3N | 0.48 | 0.71 | 0.88 | 1.39 | 1.61 | 2.09 | 2.08 | 3.50 | 6.05 | 7.85 | 7.00 | | |
| Total $\text{NH}_3\text{N/gm}$ embryo | 0.134 | 0.162 | 0.322 | 0.352 | 0.352 | 0.094 | 0.090 | 0.110 | 0.161 | 0.208 | 0.134 | | |
| Urea | | | | | | | | | | | | | |
| Embryo | 0.05 | 0.09 | 0.09 | 0.07 | 0.10 | 0.39 | 0.36 | 0.81 | 1.37 | | | | |
| Yellow and yolk sac | 0.23 | 0.69 | 0.59 | 0.79 | 0.49 | 0.21 | 0.14 | 0.11 | 0.02 | | | | |
| White | 0.06 | 0.02 | 0.05 | 0.15 | 0.16 | ... | ... | ... | ... | | | | |
| Allantoic fluid | | 0.78 | 0.40 | 0.78 | 0.22 | 2.03 | 1.77 | 1.90 | 2.22 | | | | |
| Total urea N | 0.29 | 1.54 | 1.13 | 1.79 | 0.97 | 2.63 | 2.27 | 2.82 | 3.61 | 5.46 | 8.05 | | |
| Total urea N/gm embryo | 0.73 | 0.35 | 0.535 | 0.212 | 0.212 | 0.134 | 0.11 | 0.091 | 0.096 | 0.146 | 0.160 | | |
| Uric acid | | | | | | | | | | | | | |
| Embryo | ... | 0.01 | ... | 0.02 | 0.01 | 0.12 | 0.12 | 0.29 | 0.49 | | | | |
| Yolk and yolk sac | ... | 0.23 | 0.28 | 0.21 | 0.21 | 0.06 | 0.06 | 0.05 | 0.05 | | | | |
| White | ... | 0.02 | 0.01 | 0.05 | ... | ... | ... | ... | ... | | | | |
| Allantoic fluid | ... | 0.12 | 0.12 | 0.29 | 0.07 | 0.97 | 0.77 | 0.41 | 2.56 | | | | |
| Total uric acid N | ... | 0.38 | 0.41 | 0.57 | 0.29 | 1.15 | 0.95 | 0.75 | 3.10 | 1.44 | 0.76 | | |
| Total uric acid N/gm embryo | 0.23 | 0.172 | 0.202 | 0.090 | 0.090 | 0.067 | 0.052 | 0.027 | 0.087 | 0.041 | 0.015 | | |
| Total excreted N | 0.77 | 2.63 | 2.42 | 3.75 | 2.87 | 5.87 | 5.30 | 7.07 | 12.75 | 14.75 | 15.81 | | |
| Total excreted N/gm embryo | 1.08 | 0.69 | 1.06 | 0.65 | 0.65 | 0.30 | 0.25 | 0.23 | 0.336 | 0.396 | 0.310 | | |

comparable in state of differentiation to a 5-day chick. The allantois has just been formed and has a capacity of 1.0 ml.

Nitrogen excretion. The data for ammonia, urea and uric acid as recorded for the several fractions of the egg contents are set down in table 1. Although there is variability in the results pertaining to the 4 youngest embryos because of the small quantities of material being measured, the pattern of reliance on ammonia and urea throughout the developmental period seems clear. The nitrogen excreted per gram of embryo wet weight has been calculated after subtracting the values which were found in the unfertilized egg (an average of 4 measurements). From this set of figures it would appear that the ammonia concentration remains quite constant throughout development; urea is evidently synthesized at a greater rate early in development and less in the later stages; rate of uric acid production is highest at the beginning and declines progressively as the embryo matures, as does the rate of accumulated waste nitrogen of all types. If excreted nitrogen represents deaminated protein whose carbohydrate skeleton is used for combustion, it is apparent that early in development protein serves as an energy source to a greater extent than at any later time.

Because of the relative constancy of ammonia concentration in the entire egg (4.67 mg% in the embryonic tissues) it would appear that ammonia should not in actuality be regarded as a waste product but rather as a metabolite which may have either of two fates: (1) incorporation in the urea or uric acid molecule or (2) resynthesis as protein of the embryo or its membranes. Embryonic tissues of the alligator have, then, tolerance for an inordinately high concentration of ammonia. Hopping ('23) has reported for the adult alligator that approximately 70% of the excreted nitrogen is in the form of ammonium salts, about 10% as uric acid and the balance as urea. She states that "gaseous nitrogen of the blood was found to be higher than in any animal so far reported"; however, ammonium nitrogen of the blood was only moderately high, 2.1 mg per cent.

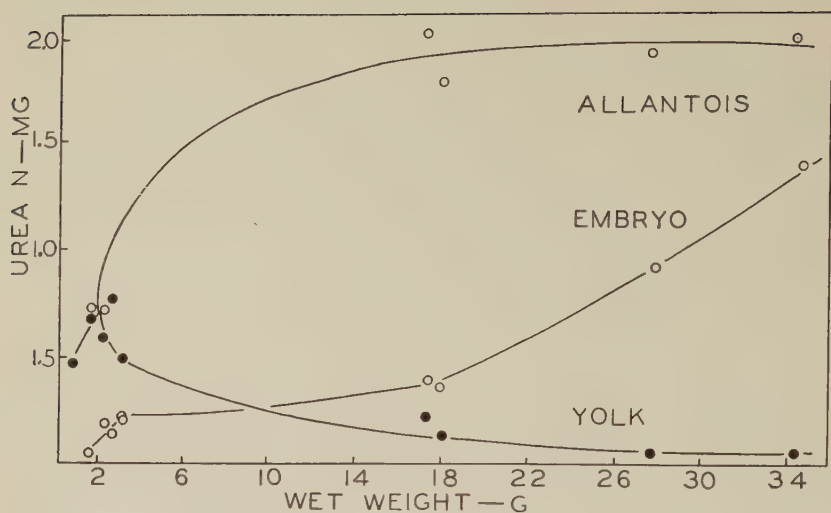


Fig. 1 Accumulation of urea nitrogen by alligator embryos from three weeks of age to hatching. Curves represent content of allantois, embryo and yolk plus yolk sac, respectively. Ordinate: urea nitrogen, mg; abscissa, embryo wet weight, grams.

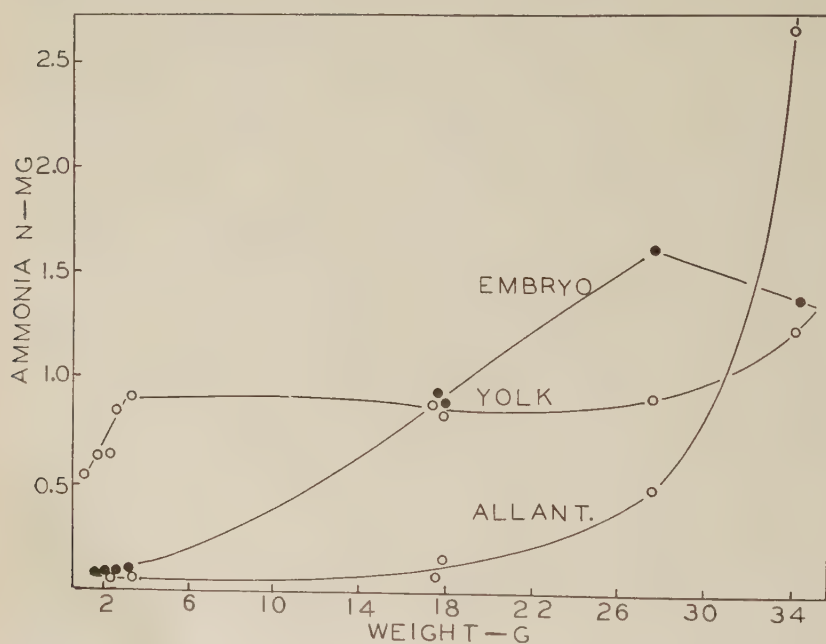


Fig. 2 Accumulation of ammonia nitrogen by embryonic tissues, yolk plus yolk sac, and allantois of alligator embryo. Ordinate: ammonia nitrogen, mg; abscissa: embryo wet weight, grams.

Data presented in figure 1 show the importance of the yolk sac in early urea synthesis and storage, with a shift to the allantois when this organ develops. The pattern of ammonia accumulation, figure 2, suggests that early in development the urea and uric acid mechanisms are adequate for its removal, but later, that a large portion of the accumulated waste nitrogen is in the form of ammonia. Since the concentration in the embryo is nearly constant throughout development, it would appear that ammonia is stored as a waste product in the allantois. Since the urea and uric acid mechanisms are plainly present, it is apparent that optimal conditions for their operation are absent toward the end of development and, we should infer from Hopping's findings ('23) that a similar situation is also true in the adult.

DISCUSSION

The observation of the importance in excretion of the yolk sac reported above for the alligator embryo confirms the observation of a similar phenomenon in the black snake. Reliance on accumulation of urea in early stages of development demonstrates the early operation of a urea mechanism; although relatively more waste nitrogen appears as urea than as uric acid, the means for synthesizing the latter are, none the less, present. The thesis of Needham ('31, '50) that nitrogen is eliminated first as NH_3 , then as urea and finally as uric acid, is not substantiated. A more obvious interpretation of the data would appear to be that ammonia is an inevitability resulting from hydrolysis and deamination of proteins; it is an end product of nitrogen metabolism, only insofar as it may exceed the capacity of the urea and uric acid mechanisms, or the capacity of the embryo to reincorporate it into tissue proteins. The reason for such failures must be sought in altered intracellular requirements for optimal performance of the respective enzyme systems. That the alternative of excessive rate of ammonia production cannot be accepted in explanation is obvious from the fact that, in this respect, the alligator is more efficient than any other animal studied,

producing only 0.35 mg waste nitrogen per gram of embryo formed. This may, however, be a reflection of the relative availability of fat and protein as energy sources in the newly laid alligator egg. Such analyses have not been made. The decline in ratio of excreted nitrogen to embryo weight from 1.08 in the embryo at three weeks of age to 0.35 at hatching suggests a greater utilization of protein as an energy source early in development. Although pursuit of this question in embryonic alligators is not feasible, work is in progress on more readily obtainable reptiles and the chick, all of which exhibit the same phenomenon.

SUMMARY

1. The alligator embryo during a developmental period of approximately two months produces 15.28 mg waste nitrogen, 0.35 mg nitrogen per gram of embryo formed.

2. Of the waste nitrogen, 46.5% appears as ammonium salts, 46.2% as urea and 7.3% as uric acid.

3. The yolk sac functions as an excretory organ prior to allantoic development and the stored urea shifts from the yolk to the allantois at this time.

4. Urea and uric acid are synthesized from the beginning of development, so that ammonia cannot be regarded as the earliest waste product in a succession.

5. Evidence is presented which suggests that protein may be the preferred source of energy in early development.

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MITOCHONDRIAL AND CYTOPLASMIC PROTEASE ACTIVITY IN SEA URCHIN EGGS¹

R. MAGGIO

*Laboratory of Comparative Anatomy, The University
of Palermo, Italy*

Previous investigations from this Laboratory (Ceas, Impellizzeri and Monroy, '55; D'Amelio, '55; Giardina and Monroy, '55; Monroy, '50; Monroy and Monroy-Oddo, '51) have given evidence of a process of re-arrangement of some proteins of the sea-urchin egg taking place as a result of fertilization. The possibility has also been considered that as a part of this process end groups of some proteins may be split off (Ceas, Impellizzeri and Monroy, '55). Indirect support to this view comes from the earlier findings of Örström ('42), recently confirmed by Ricotta ('56), of a slight but significant increase of the non-protein amino-nitrogen during the very first minutes following fertilization. On the other hand the activation of proteases immediately after fertilization has been reported by Lundblad ('49, '50, '52, '53, '54a,b); this activity subsides about 30 minutes after fertilization. According to Lundblad, in the unfertilized egg the inactive precursors of three proteolytic enzymes are present and their activation follows immediately upon fertilization. A further enzyme is active already in the unfertilized eggs and does not change upon fertilization. In his investigations Lundblad made use primarily of the viscosimetric method using gelatin as substrate. Lundblad also made the suggestion that some of these proteases may be localized in the cytoplasmic granules. Protease-activity in the cyto-

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plasmic granules of the *Arbacia* egg has in fact been reported by Woodward ('49). In general it is known that proteases are present both in the ground cytoplasm and in the mitochondria. The extraction procedure used by Lundblad, however, did not allow him to distinguish between cytoplasmic and mitochondrial enzymes.

It appeared indeed interesting to investigate whether cytoplasmic and mitochondrial proteases behave differently upon fertilization. This especially in view of the suggestion that mitochondria may take an active part in the early reactions of the egg upon fertilization (Monroy, '53). The present investigations have shown indeed an entirely different response of the two activities upon fertilization.

MATERIAL AND METHODS

Eggs of *Paracentrotus lividus* were obtained by shaking the removed gonads in sea-water. They were then filtered through cheese-cloth and washed twice with sea-water. Sperm were also obtained directly from the gonads.

For each experiment the same egg suspension was used; one part of it was left unfertilized while the rest was inseminated and samples collected out of it at different time intervals after fertilization (from three to 30 minutes).

The eggs were freed of their jelly-coat by acidification of the sea-water to pH about 5.0 and collected by low-speed centrifugation. For the preparation of the mitochondria the technique of Witter et al. ('55) was used with slight modifications. The eggs were homogenized in the cold in 0.44 M sucrose made up in 0.1 M citrate buffer at pH 6.3, containing Versene at the final concentration of $5 \cdot 10^{-4}$ M, using a glass homogenizer with teflon pestle. The homogenate was first centrifuged at $1500 \times g$ for 10 minutes in the cold and then from the supernatant the mitochondria were sedimented at $20000 \times g$ for 30 minutes in the cold. The sediment was resuspended and recentrifuged twice in 0.25 M sucrose made up as above. The final sediment was suspended in glycerophos-

phate buffer according to Duspiva ('39). The mitochondria prepared by this technique are, however, contaminated mainly with pigment granules. A greater purification can be achieved by the procedure described by Monroy ('57) which briefly consists in resuspending the mitochondria pellet in 1.0 M sucrose and centrifuging it again at high speed. In this way the pigment will float at the surface of the sucrose while the mitochondria are settled at the bottom of the tube. From these preparations samples were occasionally taken and examined in the electron microscope as described elsewhere (Monroy, '57).

In the experiments in which the activity of the total homogenate was to be studied the eggs were directly homogenized in the glycerophosphate buffer and the total homogenate used for the enzymatic assay. For the study of the mitochondria-free cytoplasmic fraction the eggs were homogenized in 0.5 M sucrose in 0.015 M phosphate buffer, pH 6.8. The homogenate was then centrifuged once for 40 minutes at $20000 \times g$ in the cold to remove the mitochondria and the supernatant was used for the determination of the protease activity.

Protease activity was determined according to Duspiva ('39). The mixture of 0.1 cm³ of the homogenate, or mitochondria-free cytoplasmic fraction or mitochondria suspension + 0.1 cm³ of buffer (according to Duspiva, '39) at the desired pH + 0.1 cm³ of the casein substrate was incubated for 30 minutes at 30°C. The reaction was stopped by the addition of 1.0 cm³ of distilled water and 2.0 cm³ of 5% trichloroacetic acid. In the zero time tubes the trichloroacetic acid was added before the substrate. Blanks of casein without egg material and of the latter without casein were run at the same time. After a few hours standing in the refrigerator, the precipitate was removed by centrifugation and the amount of split substrate determined in the supernatant by the Folin reagent: the intensity of the colour was read in the Beckman Spectrophotometer at 700 m μ against a standard of Tyrosine. The activity has been expressed as μ M of Tyrosine released *per* 10 mg of total N in the preparation after 30 minutes of in-

cubation. Total N was determined by direct Nesslerization after combustion.

RESULTS

The experiments reported here have been run at pH between 4.5 and 8.3. Activity has been found constantly only between pH 5.4 and 5.9, with a maximum at pH 5.4. At pH other than those, no activity or a very slight one has been found. These latter experiments therefore have been omitted from the tables.

Protease activity in the total homogenates

Unfertilized eggs. A test was first made of whether or not any change occurs in the protease activity of unfertilized eggs which had been standing over a period of one hour, i.e. over a period twice as long as the one used in the experiments with fertilized eggs. In these experiments one sample of eggs was collected immediately after the second washing (and this was taken as the zero time) and then two more samples after 30 and 60 minutes were collected. Five such experiments were done. The mean values were, for the zero time samples $4.79 \mu\text{M}$ Tyrosine and for the 60 minutes samples $4.33 \mu\text{M}$ Tyrosine. The slight difference is statistically not significant ($t = 0.51$, $P < 0.70$ and > 0.50). Hence, practically no change seems to take place in the protease activity of the unfertilized eggs over a period of one hour.

Changes following fertilization. The results of this series of experiments are collected in table 1. The data show a rise of the protease activity beginning during the first 5 minutes after fertilization and reaching its maximum between 5 and 15 minutes. The statistical significance of this rise has been calculated with the χ^2 (of Pearson) and has given a value of $P \sim 0.001$. A slight decrease of the activity then appears to take place at about 30 minutes.

To distinguish between cytoplasmic and mitochondrial activities, the extracts were then fractionated as described before and the activity of each fraction tested separately.

Protease activity of the cytoplasmic fraction. The results of these experiments are collected in table 2. As it can be seen, they duplicate satisfactorily those obtained with the total extracts with the only difference that here somewhat higher activities have been observed. Furthermore both in the experiments with the cytoplasmic fraction and in those with the isolated mitochondria a larger variability of the absolute values between different batches of eggs has been observed.

TABLE 1

Protease activity in the total homogenate of eggs of Paracentrotus lividus.
 μM of Tyrosine/10 mg total N/30 minutes of incubation

| EXP. NO. | pH | UNFERTILIZED | MINUTES AFTER FERTILIZATION | | |
|----------|-----|--------------|-----------------------------|------|------|
| | | | 5 | 15 | 30 |
| 1 | 5.4 | 3.17 | 4.24 | 3.76 | 3.17 |
| 2 | 5.4 | 4.42 | 4.49 | 4.63 | 3.89 |
| 3 | 5.4 | 2.79 | 4.03 | 4.21 | 3.00 |
| 4 | 5.7 | 2.39 | 3.73 | 4.16 | 1.85 |
| 5 | 5.7 | 3.70 | 4.34 | 3.55 | 4.33 |
| 6 | 5.7 | 2.56 | 3.56 | 3.64 | 2.85 |
| 7 | 5.9 | 2.09 | 2.49 | 2.57 | 3.11 |
| 8 | 5.9 | 3.57 | 3.61 | 2.01 | 2.98 |
| 9 | 5.9 | 1.57 | 2.07 | 2.93 | 2.49 |
| 10 | 5.9 | 1.48 | 2.29 | 1.60 | 1.78 |

TABLE 2

Protease activity in the mitochondria-free cytoplasm of eggs of Paracentrotus lividus. μM of Tyrosine/10 mg total N/30 minutes of incubation

| EXP. NO. | pH | UNFERTILIZED | MINUTES AFTER FERTILIZATION | | |
|----------|-----|--------------|-----------------------------|------|------|
| | | | 5 | 15 | 30 |
| 1 | 5.4 | 2.37 | 2.68 | 2.94 | 2.81 |
| 2 | 5.4 | 3.72 | 5.09 | 6.24 | 5.51 |
| 3 | 5.4 | 5.00 | 5.76 | 6.56 | 6.32 |
| 4 | 5.4 | 7.05 | 7.52 | 6.69 | 6.78 |
| 5 | 5.4 | 6.50 | 7.88 | 7.43 | 8.17 |
| 6 | 5.4 | 4.60 | 7.76 | 4.00 | 4.97 |
| 7 | 5.9 | 2.30 | 2.69 | 4.34 | 4.33 |
| 8 | 5.9 | 3.46 | | 3.08 | 2.87 |
| 9 | 5.9 | 2.17 | 3.56 | 2.95 | 3.35 |

Protease activity in mitochondria. The data collected in table 3 show a drop of activity of the mitochondrial protease during the first few minutes following fertilization, followed by an increase between 10 and 15 minutes and finally, at about 30 minutes, values somewhat lower than in the unfertilized eggs were obtained.

TABLE 3

Protease activity in mitochondria of eggs of Paracentrotus lividus
 μM of Tyrosine/10 mg total N/30 minutes of incubation

| EXP. NO. | pH | UNFERTILIZED | MINUTES AFTER FERTILIZATION | | |
|----------|-----|--------------|-----------------------------|------|-------|
| | | | 5 | 15 | 30 |
| 1 | 5.4 | 8.74 | 7.09 | 8.98 | 6.75 |
| 2 | 5.4 | 4.96 | 3.90 | 3.99 | 3.88 |
| 3 | 5.4 | 10.68 | 8.13 | 8.16 | 7.70 |
| 4 | 5.4 | 13.87 | 12.96 | | 10.36 |
| 5 | 5.4 | 9.23 | 7.88 | 8.56 | 8.52 |
| 6 | 5.4 | 9.38 | 7.06 | 8.41 | 7.94 |
| 7 | 5.4 | 3.30 | 2.20 | | |
| 8 | 5.4 | 5.29 | 1.86 | 3.63 | 3.43 |
| 9 | 5.7 | 8.37 | 4.11 | 6.29 | 5.35 |
| 10 | 5.9 | 5.35 | 4.05 | 6.10 | 5.12 |
| 11 | 5.9 | 5.66 | 4.71 | 5.18 | 3.51 |
| 12 | 5.9 | 7.85 | 4.27 | 6.72 | 4.68 |

DISCUSSION

The results of the present experiments indicate the presence of a protease activity in the mitochondria and in the ground cytoplasm of the unfertilized egg of *Paracentrotus lividus*. The activity both of the cytoplasmic and of the mitochondrial protease shows up in the same range of pH, i.e. between pH 5.4 and 5.9. Pending further studies it cannot be stated at present whether they are the same or different enzymes.

During the first few minutes following fertilization the protease activity in the whole homogenates of eggs increases, thus confirming the earlier observations of Lundblad ('49, '50, '52, '53, '54a,b).

When, however, the activity is tested separately in the mitochondria and in the cytoplasm after removal of the

mitochondria, it is found that while in the former it undergoes a decrease, in the latter it exhibits an increase. This suggests that the activity of the cytoplasmic protease undergoes an actual temporary increase immediately after fertilization. In fact should the overall change depend on a leakage of enzyme from the mitochondria into the cytoplasm (spontaneous or artificial, as will be discussed presently), the total activity should remain constant.

In the mitochondria on the other hand the protease activity appears to undergo cyclical changes, being low immediately after fertilization, then going up slightly at about 15 minutes (sperm-aster stage) and then decreasing again at 30 minutes (nuclear-streak stage). It is difficult at present to suggest any interpretation for these changes. Evidently the possibility of a differential breakage or damage of mitochondria during the homogeneization before and after fertilization must be borne in mind (see Monroy, '57). Although the electron microscope pictures fail to indicate any difference between preparations of mitochondria from unfertilized and fertilized eggs, there is the possibility that after fertilization the condition of the mitochondria, viz. of the mitochondrial membrane, may undergo cyclical changes of susceptibility, just as it is known to occur in the cortex of the egg. This may result in a differential release of enzyme during the preparation in the different stages. This possibility seems to be sufficiently interesting to deserve further investigations. Should on the contrary the above hypothesis be disproved, then the changes in the activity of the protease observed in the mitochondria would depend on changes in the activity of the enzyme itself.

In any event these results are suggestive of a process of re-arrangement taking place in the mitochondria of the egg upon fertilization.

SUMMARY

A protease activity (at pH between 5.4 and 5.9) has been found in the ground cytoplasm and in the isolated mitochondria of the eggs of *Paracentrotus lividus*. It has been found

that in the mitochondria-free cytoplasm the activity undergoes an increase at fertilization; in the mitochondria on the contrary a decreased activity follows fertilization.

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THE ROLE OF HUMIDITY IN THE DIFFERENTIATION OF THE CELLULAR SLIME MOLDS ¹

JOHN TYLER BONNER AND MARCIA J. SHAW

Department of Biology, Princeton University, Princeton, New Jersey

ONE FIGURE

The cellular slime molds arise by an aggregation of amoebae, and the resulting cell mass often will wander some distance over the surface of the substratum before rising into the air to fruit. The fruiting body consists of a single or branched stalk made up of large vacuolate amoebae encased in a tapering cellulose sheath and a terminal sorus containing many encapsulated amoebae, or spores. During the wandering or migration stage, prior to the final differentiation of the fruiting body, a stalk may be continuously formed as is usually the case in many species and strains of *Dictyostelium* and *Polysphondylium*, while the species *Dictyostelium discoideum* (Raper, '35) is one of those forms having a stalk-free migrating cell mass.

The question to be considered here is what are the factors which cause the prolongation of the migration period, or conversely, what are the factors which induce the final differentiation? This is an old problem which was discussed by many of the earlier workers, and Potts ('02) in particular performed numerous interesting and pertinent experiments. He demonstrated for the first time that humidity plays an important role in development, although he believed the effect to be largely on stalk formation rather than on final

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spore differentiation. There is little point here in reviewing his experiments in detail; he pioneered and showed the way, although we may not be in complete agreement with all his observations today.

In recent years the experimental approach to the factors governing final differentiation was first used by Raper ('40) on the migration stage of *Dictyostelium discoideum*. He showed that there were two principal external factors which were important for the duration of migration: humidity and temperature. If a Petri dish is partially opened for a time, the migrating bodies round up preparatory to fruiting. This effect is reversible if the desiccation has not gone too far, for by controlling the time of cover removal, culmination would proceed directly in a normal fashion. The same effect was achieved by raising the temperature. The optimal temperature Raper considers to be 20° to 24°C. If the cultures are grown at 26° to 28°C or if they are grown at a lower temperature and brought to this higher range during migration, the effect in both cases is the curtailment of the migration phase. Any reduction in temperature would have the opposite effect of sustaining prolonged migration.

The problem of the duration of migration was examined from another aspect by Slifkin and Bonner ('52). They found that the concentrations of solutes in the agar directly affected the time of migration; the more dilute the agar, the longer the migration.

In this paper we shall put forth evidence to support the contention that the basic stimulus to the final differentiation is, as Potts believed, a water loss, or desiccation. This view not only conforms in general with the previous experiments just cited (for both increase in temperature and increase in solutes would cause desiccation) but with the new experiments to be presented here.

METHODS

The slime molds were all fed on *Escherichia coli*. Three different types of culture media were used: (1) A standard agar which contains 1% peptone and 1% dextrose and is

buffered with phosphate buffers to pH 6; (2) A lactose-peptone agar (from Raper, '51) which contains 0.1% of both substances; (3) Plain Bacto agar made up to 2%. (In the experiments using different concentrations of dextrose, the latter was added directly to 2% agar.)

Unless otherwise stated, the experiments were run at 22°C in a B.O.D. cabinet with circulating air. In all the experiments described here the slime molds migrated towards a 1 watt neon G.E. "glow lamp" bulb. In order to reduce reflections the incubator was lined with porous dark paper.

RESULTS

The effect of the concentration of solutes in the agar on the migration of D. mucoroides. Agar containing different concentrations of dextrose (from 0.1 to 6%) were inoculated with *D. mucoroides* and the results conformed with those found previously for *D. discoideum* (Slifkin and Bonner, '52). Namely, migration decreased with an increase in concentration of the solute. When grown on a low solute agar there was considerable variation among the different strains for 9 of the 18 tested showed prolonged migration, while the other 9 would not migrate at all. (Three strains of *D. purpureum* were also tested and two showed the typical long migration on plain agar, while the third fruited without migration).

In those strains that migrate for extended periods it was possible to obtain remarkably long sorocarps. Previously Singh ('47) had reported that his large strain of the mucoroides type (which he called *D. giganteum*) ordinarily has a stalk of 1.7 to 2.5 cm. with occasional ones as long as 3.0 cm. Using an agar of low solute content, and causing the cell masses to orient toward a light (so as to give a straight stalk), it was possible to obtain stalks as long as 22 cm. This far exceeded the distance across ordinary Petri dishes (9 cm.) and culture bottles as well as pyrex baking dishes had to be employed. The phenomenon is especially interesting when one considers the fact that the initial size of the cell mass is not abnormally

great, but it is simply that because of the long migration, the majority of the cells turn into the stalk and the final sorus is minute. (This matter will be considered in detail in a later paper).

A parenthetic note should be added here. In one of the experiments when the *D. mucoroides* had traveled 15 cm., the pseudoplasmodia migrated into a colony of bacteria that had appeared by accident. The colony became infected with amoe-

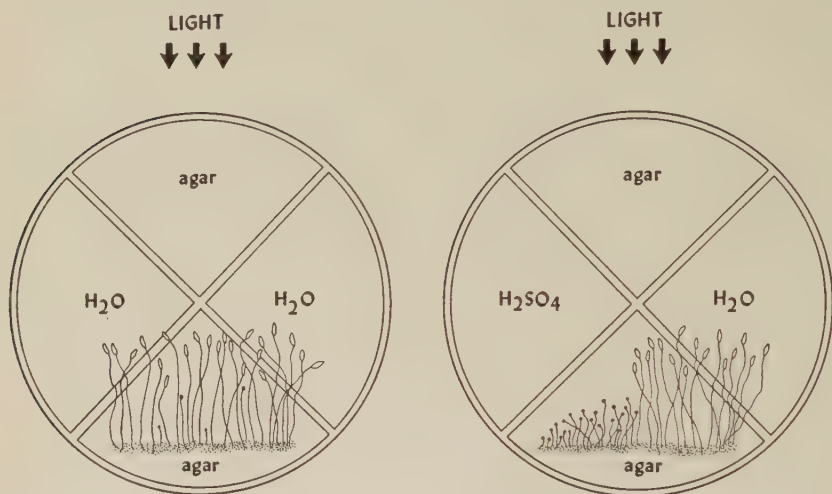


Fig. 1 A diagram illustrating the effect of humidity on the final differentiation in *D. mucoroides*. In the right hand dish, the pseudoplasmodia on the dry side sporulate early, while those on the wet side continue to migrate. In the control in the left hand dish, both sides are equally humid and migration is the same everywhere.

bae, and after a couple of days new large pseudoplasmodia started again towards the light. In the presence of such relay stations it would theoretically be possible to obtain infinite dispersion of the slime mold.

The effect of relative humidity on the duration of migration in D. mucoroides. In a chance observation it was noticed that in a particular dish the agar was drying on one side only, and on that side the pseudoplasmodia had fruited and formed mature sori sooner than on the wet side, giving shorter mature

sorocarps. This essentially is the observation of Raper ('40) and we now tested it systematically using two long-migration strains of *D. mucoroides* (DM-4, -11).

Felsen culture dishes (Petri dishes divided by raised ridges into four pie-shaped quadrants) were prepared so that the north and south quadrants contained 2% agar, the east quadrant contained distilled water, and the west quadrant contained sulfuric acid solutions giving relative humidities of a range from 20 to 95% (fig. 1). The controls consisted of reversing the acid and water quadrants in one set, and in another water was put in both quadrants. A loopful of *E. coli* and spores of *D. mucoroides* were placed in the east-west direction across the widest position of the southern quadrant containing agar, and the light was placed in a northerly position.

With the exception of the all-water controls, in every case and for both strains (DM-4, -11) there was greater migration and therefore longer sorocarps on the more humid side (fig. 1). It was impossible to observe any difference in the result using different sulfuric acid solutions; they all appeared approximately equally effective. In all-water controls the extent of migration was the same on both sides.

It is of course, impossible, in a culture dish containing sulfuric acid, agar and water, not to mention a loose fitting top, to have any idea of the regional fluctuations in humidity. The fact that the molds differentiate into spores between 95 and 100% relative humidity as well as being affected by small changes in the osmotic pressure of the medium suggests considerable sensitivity to small differences at the high end of the humidity range, and, as will be shown, this was confirmed experimentally.

It is a well known fact that a Petri dish, with its loose fitting top, will readily allow moisture to escape. In other words, the relative humidity within a Petri dish may not be at saturation, except right at the surface of the agar. Capitalizing on the fact that *D. mucoroides* is strongly phototactic, an experiment was run on low solute agar (both 0.1% lactose-

peptone agar and plain 2% agar) in which one set of dishes was on a plane with the light, so that the advancing migrating masses clung to the surface of the agar, and the other was placed six inches below and ten inches away from the light so the cell masses tended to lift up from the agar at an angle of about 30°. Of course the heavy cell masses did not remain suspended in the air but would keep falling back down to the agar at intervals and then start up again. Five separate runs were made for each condition and when the light was on a level with the dish, in 589 sorocarps, four days after inoculation, the mean per cent that had differentiated was $10.6 \pm 2.4\%$ (the standard deviation). When the light was above the culture dish and the sorocarps were primarily aerial, in 288 cases the mean per cent that had finished their differentiation was 56.3 ± 19.8 . Clearly the aerial sorocarps differentiate sooner and the only obvious difference between the two conditions is a slight difference in the humidity between the surface of the agar and a few millimeters distant.

In another experiment the molds were oriented toward the light on plain agar along the bottom of large, enclosed rectangular culture bottles (32 oz. capacity, 2 inches deep, stoppered with a cotton plug). The bottles were separated into two groups, both level with the light, but in one set the bottles were inverted so the sorocarps were "hanging from the ceiling." After a centimeter or more of migration, these inverted sorocarps tended to fall loose from the agar substratum and hang down into the air of the jar. Invariably in this condition they would soon show final spore differentiation while the controls which were right side up showed extended migration along the surface of the agar.

It should be noted that many of the earlier authors, including Potts ('02), believed that there is a negative hydrotropism in the orientation of the migrating and culminating sorocarp. During the course of the experiments with the Felsen dishes cited above some special experiments were run in which the *D. mucoroides* was innoculated in a north-south direction between the sulfuric acid and the water and the plates were kept

in an air circulating incubator in the dark. In none of the experiments was there any evidence of a hydrotropism. It must be admitted that these negative experiments do not rule out the possibility of such a phenomenon, but if it exists its role is probably limited.

Humidity relations in other species: We have repeated some of the above experiments with *Polysphondylium violaceum* and *P. pallidum* and find that they respond as *D. mucoroides*. Again there is a considerable variation among strains, but in long migration strains of both species there is extended migration in the presence of high relative humidity and low solute agar. In the case of the small *P. pallidum* stalks have been produced with a length of 4 cm, and in the larger *P. violaceum* the maximum length achieved is 8 cm. The interesting thing is that while characteristically numerous orderly whorls appear near the tip, there are often small whorls or individual branches occurring the length of the elongate stalk. This certainly would seem to point up the effectiveness of spore dispersal in the *Polysphondylium* type fruiting structure, for there may be sori strung out over a distance as great as 8 cm.

Good support for the arguments presented here may be found in the recent investigations of Wittingham and Raper ('55) on the conditions affecting fructification of *Dictyostelium Polycephalum* sp. nov. (Raper, '56). They showed that in this species the relative humidity existing within a culture chamber is a most important factor. Migration continues indefinitely and sorocarps will not form in Petri dishes with conventional glass covers, although normal differentiation will occur in plates with porous covers (e.g. unglazed porcelain). They were able to show that the function of the porous cover was to allow the escape of water vapor.

The effect of temperature: If the effect of an increase in temperature upon the cessation of migration observed by Raper ('40) is to be interpreted in terms of humidity, than we must expect that only an increase in temperature will be important, and a constant high temperature should have no in-

hibiting effect on migration. The reason for this is that raising the temperature momentarily lowers the relative humidity, but a sustained high temperature over an agar surface should keep a constant high humidity, provided there is not too great a moisture escape by evaporation.

Using constant conditions, with plain agar which favors migration, and making special efforts to reduce evaporation, it was possible to get prolonged, normal migration of *D. discoideum* at 28°C, and the two strains of *D. mucoroides* (DM-4, -11) that were tested behaved normally at 32°C. Temperatures above the limits for either species resulted in no growth or development of any kind.

We also found that if a high solute agar was used (buffered 1% dextrose-peptone) then at high temperature the migration was completely inhibited, which brings our observations in line with those of Raper ('40).

DISCUSSION

If, as the evidence indicates, a decrease in humidity is the prime factor controlling the final sporulation, it may be well to examine the matter from an ecological point of view.

Feeding and aggregation must occur in water. That is any stage which involves the free movement of separate amoebae requires an aqueous medium. It is conceivable, on the other hand, that both for the formation of spores and for their more effective dispersal, that an aerial position is advantageous. The migration stage removes the cell mass from moist to drier regions, and it is assisted or guided in this process by being oriented to light and to heat. If a considerable area is wet then migration continues and stops only when there is a slight decrease in the humidity. It may of course be that for dispersal it is only important to remove the spores some distance from the feeding-aggregation site, and the reason that a slight decrease in humidity stimulates sporulation is that any drastic humidity decrease would prevent sporulation and kill the delicate, actively moving amoebae. Therefore, a slight decrease is a warning signal and sporulation ensues rapidly before the dry

air can do any damage. The suggestion made here, then, is that the effect of small changes in humidity is a defense mechanism in which the slime mold may capitalize on the migration distance gained (which is important for effective dispersal) before any irreparable loss might occur.

ACKNOWLEDGMENTS

The authors are indebted to Drs. K. B. Raper and B. M. Shaffer for reading the manuscript and for providing some helpful suggestions.

SUMMARY

It is suggested that all the known external stimuli of final differentiation in the cellular slime molds can be explained in terms of a decrease in humidity. Not only is a humidity decrease directly effective, but also an increase in the solute content of the agar (which would actively desiccate) promotes sporulation, as well as an increase in temperature (which lowers the relative humidity). Constant high temperatures which will not affect the humidity when evaporation is prevented, do not induce spore differentiation.

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A COMPARATIVE STUDY OF SPECTRAL SENSITIVITY IN TADPOLES AND ADULT FROGS ¹

DONALD KENNEDY ^{2,3}

The Biological Laboratories, Harvard University

THREE FIGURES

The role of photosensitive pigments in the visual process has been demonstrated most satisfactorily by experiments in which sensitivity (reciprocal of threshold) is measured as a function of wavelength. The spectral sensitivity curves expressing these data are then compared with the absorption spectra of extracted photopigments; an agreement as to shape and position of the maximum constitutes strong evidence implicating the pigment in primary visual events. In the human eye, for example, the peripheral sensitivity (dark adapted) matches well the absorption spectrum of the rod pigment rhodopsin, both having maxima at about 500 m μ (Stiles and Smith, '44).

In the frog, Wald ('46) has demonstrated an interesting biochemical metamorphosis involving the visual pigments. The tadpole eye contains Vitamin A₂ and porphyropsin (absorption maximum 522 m μ), a rod pigment with its carotenoid chromophore derived from Vitamin A₂; after metamorphosis, the frog — like other terrestrial vertebrates — is found to have Vitamin A₁ and rhodopsin. This situation thus presents an unparalleled opportunity to study both visual systems in the same animal species.

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²Pre-doctoral research fellow of the National Science Foundation.

³Present address: Department of Zoology, Syracuse University, Syracuse, New York.

In the present experiments, the electroretinogram was used to determine the spectral sensitivity of tadpoles and frogs. The electroretinogram is a slow potential which can be recorded from the cornea of the eye; it represents the massed discharge of a number of retinal units whose identity is uncertain. Nevertheless, it has proven a satisfactory index of sensitivity if properly employed. Early work using this technique on the frog by Piper ('11) and Chaffee and Hampson ('24) suggested the involvement of rhodopsin in rod sensitivity, but the state of adaptation was not kept constant. Granit and Munsterhjelm ('37) and Granit and Wrede ('37) determined spectral sensitivity in dark and light adapted frogs respectively. These curves clearly demonstrate the Purkinje phenomenon, and give a good idea of the location of sensitivity maxima. Unfortunately, however, these authors presented the animals with a series of wavelengths of equal energy content, and then plotted the height of the response as a function of wavelength. It has been pointed out by Hecht and Pirenne ('40) that such curves do not actually represent the absorption spectra of visual pigments. The method assumes a linear relationship between stimulus intensity and the amplitude of the physiological response; but the relationship is in fact *not* linear, but sigmoid.

A second objective of the experiment reported here was to compare the photopic sensitivity of tadpoles and frogs with the existing chemical data on cone pigments. Wald, Brown and Smith ('55) have extracted iodopsin from the cones of the chicken retina; this pigment is based upon the same carotenoid as that of rhodopsin, but it is combined with a different protein. Although iodopsin is apparently only one of several cone pigments in the human retina (Wald, '45), it is not known whether one or several pigments determine cone sensitivity in other animals. Wald, Brown and Smith ('53) were also able to synthesize a second cone pigment, called cyanopsin, by combining the carotenoid derived from Vitamin A₂ with the protein from chicken cones. This pigment, which absorbs maximally at 620 mμ, has never been extracted from a living

animal; but there is good reason to expect that it may be the cone pigment of animals which possess the Vitamin A₂-porphyropsin visual system. Careful photopic sensitivity measurements were made on frogs and tadpoles in an attempt to provide answers to these questions. A preliminary report of some of the results has appeared elsewhere (Kennedy, '55).

METHODS

The frogs (*Rana pipiens*) used in these experiments were injected with 2 cc of a solution of d-tubocurarine chloride (0.15 mg/cc). This caused paralysis but did not affect the retinal response. Stable electrical responses could be obtained from such preparations for as long as 24 hours. Tadpoles (same species) were anaesthetized with 3% Urethane.

The electroretinogram was recorded through cotton wick electrodes, one placed on the cornea and the second (an indifferent electrode, grounded through the pre-amplifier) on the skin surface. The wicks connected through Ringer-filled pipettes to silver chloride electrodes. A capacity-coupled Grass P-4 preamplifier and oscilloscope were used.

The light source was a 300-watt tungsten filament lamp, operated with a voltage stabilizer. The intensity of the stimulus was varied with a pair of opposed annular wedges, built in the Jefferson Physics Laboratory of Harvard University and loaned through the courtesy of Professor George Wald. These provided an intensity range of six logarithmic units.

Monochromatic light was produced by the use of narrow-band interference filters (Baird Associates). These were combined with Corning and Wratten filters in order to remove unwanted bands of transmission. The transmission of all filter combinations was measured with a Beckman DU spectrophotometer.

Collimated light from the source was passed through the filters and a photographic shutter. A pair of 15 cm focal length biconvex lenses focused the light, passed it through the wedges, and recollimated it on the other side. The beam was reflected

downwards by a mirror, and a third short-focus lens produced a spot of homogeneous intensity which filled the pupil of the preparation.

The optical system was calibrated by placing a thermopile in the position of the eye, and recording its output with a sensitive galvanometer. Measurements of intensity were made at a number of wedge settings for each wavelength used. At low intensities, below the sensitivity range of the thermopile, relative energies were measured with a photocell, amplifier and oscilloscope.

In measuring spectral sensitivity, the large, cornea-positive potential of the electroretinogram (the b-wave) was used as an index of sensitivity. A certain b-wave amplitude was arbitrarily selected as a "criterion response"; the stimulus intensity just necessary to elicit a b-wave of this amplitude was then found at each wavelength, and the reciprocal of that intensity value was plotted against the wavelength. In determining scotopic sensitivity, thoroughly dark adapted animals were used, and intensities were kept low. Stimulus duration was $1/50$ sec., and at least 30 seconds were allowed to intervene between stimuli. In measurements of photopic sensitivity, animals were light adapted before the experiment. The response at some wavelength was then measured repeatedly until sensitivity became constant. Determinations of sensitivity at each wavelength were then made while the eye was on the "cone plateau" of dark adaptation. In all cases, determinations of sensitivity were made at one wavelength before and after the experiment to insure that sensitivity had remained constant during the period of measurement.

In general, two determinations of spectral sensitivity were made on each animal, reversing the order of wavelength presentation for the second experiment. An average of these two determinations was then used as the spectral sensitivity curve for that animal.

In some experiments, animals were presented with equal-energy spectra, and the responses at each wavelength recorded

on film. This method is useful to verify the position of maximum sensitivity, but does not yield a function comparable to actual spectral sensitivity curves.

RESULTS

Average spectral sensitivity curves for light and dark adapted frogs are shown in figure 1. In each case, these rep-

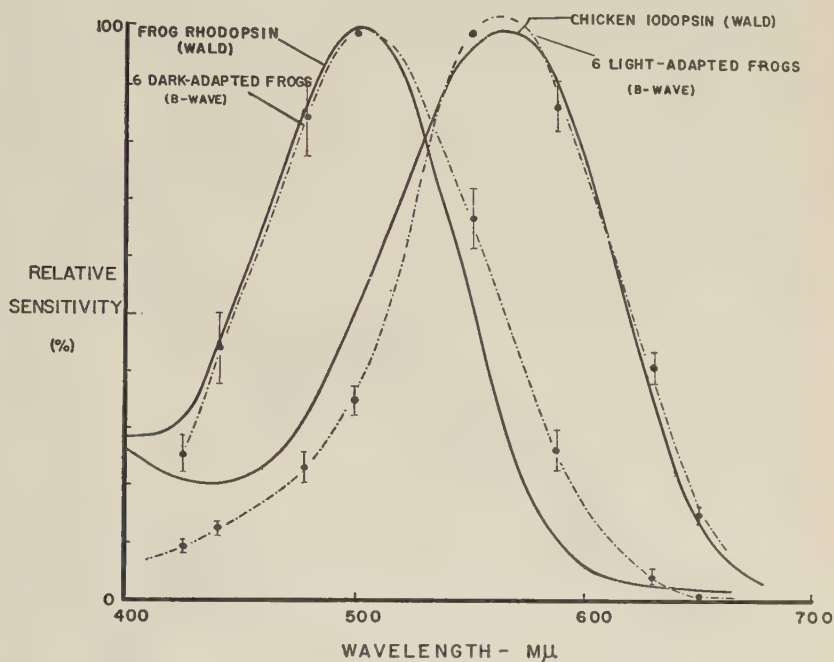


Fig. 1 Spectral sensitivity curves for dark and light adapted frogs (dotted lines), compared with Wald's absorption spectra for frog rhodopsin and chicken iodopsin (solid lines). Each curve is an average of data from six animals; standard error is indicated by vertical lines through the points.

resent the average of determinations on 6 animals; the standard error of each point is plotted. Absorption spectra of frog rhodopsin and of chicken iodopsin, both from the data of Wald, are included for comparison. (Wald, '55; Wald, Brown and Smith, '55).

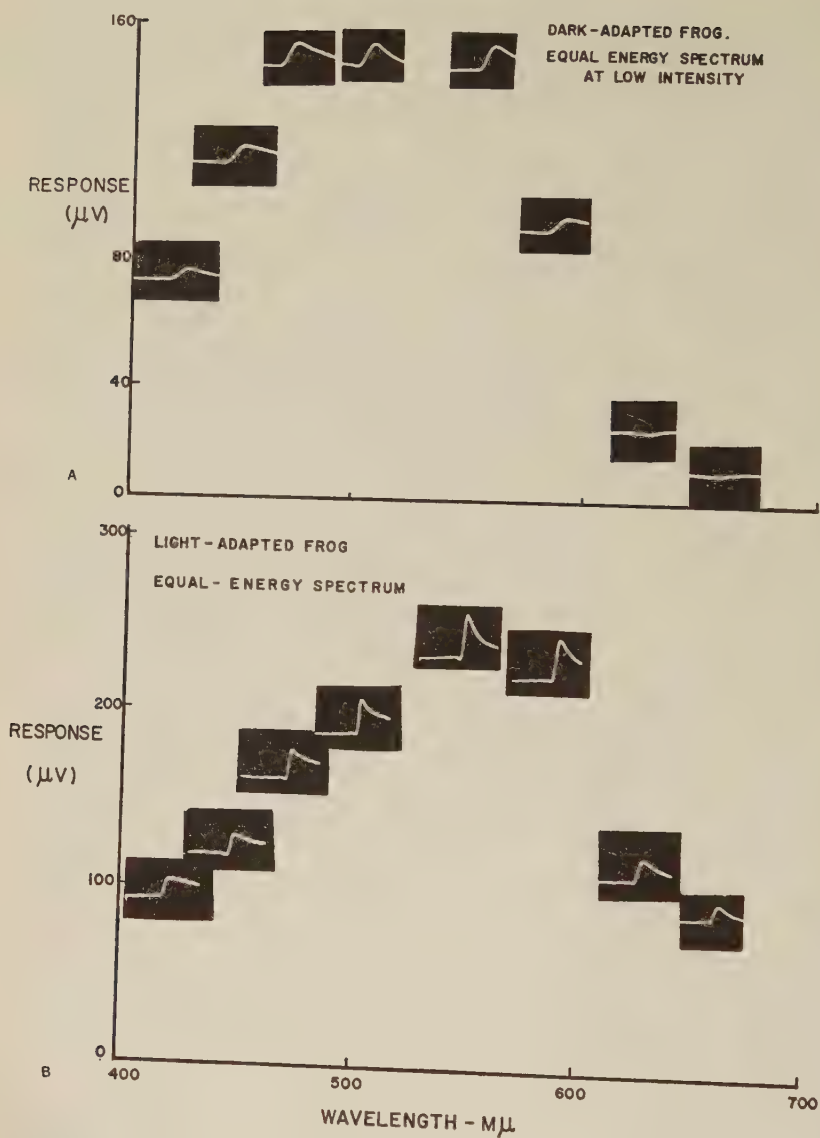


Fig. 2 Responses of the frog's eye to stimulation at equal energies. A. Dark adapted; B. Light adapted. Stimulus duration 1/25 second; stimulus intensity in B 100 times that in A.

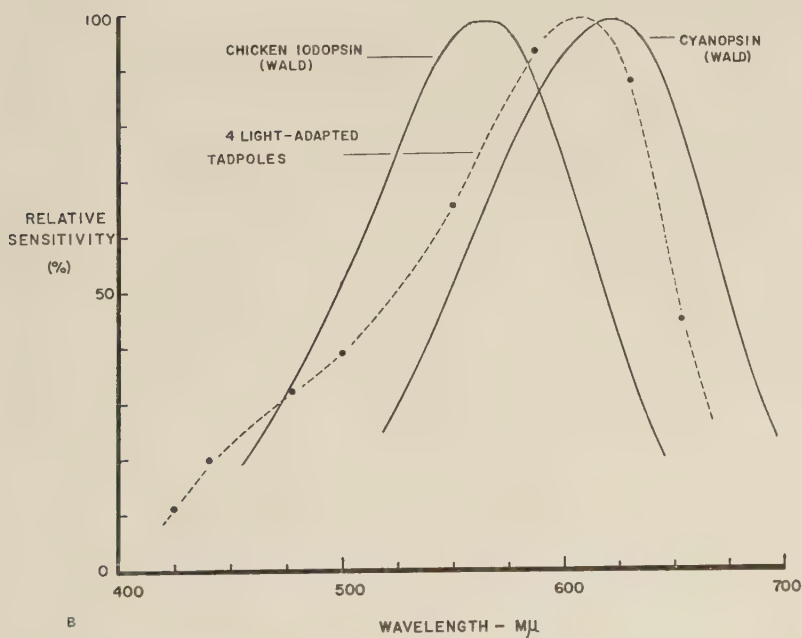
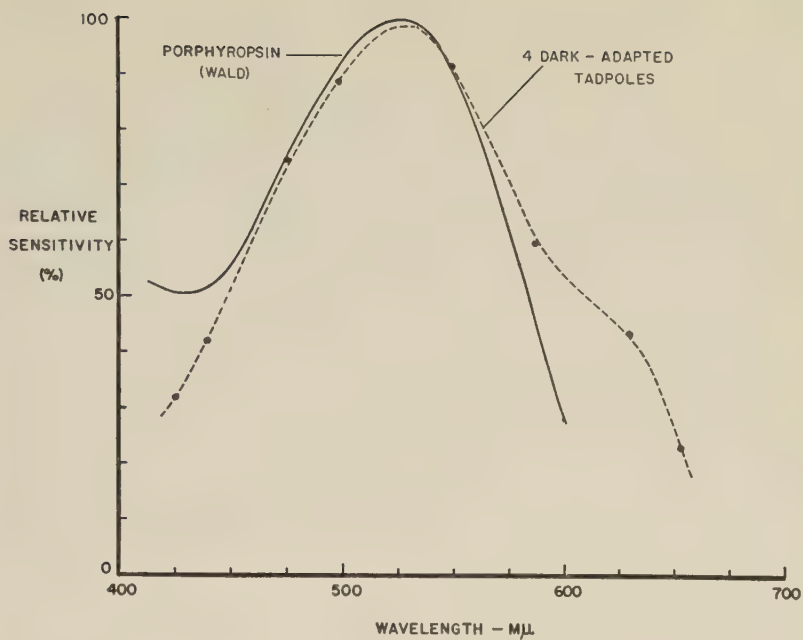


Fig. 3 Spectral sensitivity curves for the tadpole. A. Dark adapted, compared with Wald's absorption spectrum for porphyropsin (solid line). B. Light adapted; compared with absorption spectra for cyanopsin and iodopsin (solid lines).

Agreement appears to be good between rod sensitivity and the absorption of rhodopsin, and between cone sensitivity and iodopsin absorption. The positions of the respective maxima are approximately the same, and the shapes of the curves closely similar. The major discrepancy occurs below 430 m μ , where the lens begins to absorb selectively (Milkman and Kennedy, '55).

The positions of the sensitivity maxima were verified by experiments in which the dark or light adapted animal was presented with an equal-energy spectrum, and the resulting responses at each wavelength photographed at equal amplification. Figure 2 shows the amplitude of such responses plotted against wavelength for dark adapted (2 A) and light adapted (2 B) eyes.

Figure 3 shows spectral sensitivity curves for the tadpole's eye, determined in the same way as those for the frog in figure 1. In the dark adapted eye, agreement is good with the absorption of the rod pigment porphyropsin, though the high intensities necessary to elicit the electroretinogram in these animals apparently brings some photopic units into play, accounting for the excessive long-wavelength sensitivity. In the light adapted tadpole (fig. 3 B), the maximum sensitivity is displaced far into the red — almost, but not quite, to the position of maximum absorption of cyanopsin.

DISCUSSION

These results affirm the agreement of scotopic sensitivity in the frog with the absorption of rhodopsin. Further, the sensitivity distribution of light adapted animals suggests that cone sensitivity is probably determined solely by the cone pigment iodopsin. Granit ('41 b), recording from single ganglion cells in the frog retina, found elements which showed narrow-band spectral sensitivity; he called such elements "modulators." Granit attributed their behavior to the presence of several specific cone types, and felt that the light adapted spectral sensitivity function (the "photopic dominator") represented the integral of this modulator activity.

The spectral sensitivity of these modulator elements, however, was derived by procedures involving difference spectra between averaged curves, and selective adaptation. The complex, shifting discharge pattern of the retinal ganglion cell is well known (see Kuffler, '53), and it seems quite possible that such effects as those found by Granit could arise through inhibitory interaction between rod and cone pathways. It is doubtful whether the frog possesses a color-discrimination ability (Walls, '42); thus there is no need to postulate such "primaries" to adhere to color vision theory. In the case of the human eye, where a color discrimination ability obviously exists, spectral sensitivity data clearly show that iodopsin cannot be solely responsible for cone sensitivity. In the frog, however, the close agreement between iodopsin absorption and photopic sensitivity suggests that here one type of cone, with a single pigment, is functioning.

The experiments on tadpole spectral sensitivity support the conclusion, already reached on the basis of pigment extractions, that the larval frog exchanges its porphyropsin for rhodopsin during metamorphosis. In the course of these experiments, it appeared that the spectral sensitivity did not change to that characteristic of the adult animal until the very end of metamorphosis, when tail resorption had begun. A larger series of animals would be necessary to determine the exact time at which the change occurs.

The results further suggest strongly that cyanopsin is the functional cone pigment of the tadpole. Granit ('41a) has presented evidence from microelectrode studies on the tench and the turtle that these animals, too, possess a strong red sensitivity. In the present data, the discrepancy between the maximum absorption of cyanopsin and the peak sensitivity of the light adapted tadpole's eye amounts to about 10-15 m μ (see fig. 3B). The data of Wald reveal that the tadpole eye, in addition to its porphyropsin, contains a small amount of rhodopsin as well, resulting in a slight displacement of the absorption maximum of rod-pigment extracts to shorter wave-

lengths than those typical for pure porphyropsin. The discrepancy would not be noticeable in the scotopic sensitivity function (fig. 3A), since the shift is only a few m μ . The presence of a small amount of iodopsin, however, would cause a marked shift in photopic sensitivity from the position expected on the basis of pure cyanopsin. It is quite likely that this "impurity" of tadpole visual pigments lies behind the observed discrepancy.

SUMMARY

1. The data presented here verify the observation that in the metamorphosis from tadpole to frog, visual pigments belonging to the Vitamin A₂ system are replaced by another pair whose carotenoid chromophores are related instead to Vitamin A₁.

2. In the adult frog, photopic sensitivity appears to depend solely upon the cone pigment iodopsin. This finding argues against the hypothesis of Granit that several different cone types combine to produce the photopic spectral sensitivity function.

3. In the tadpole, photopic sensitivity data support the view that cyanopsin, a red-sensitive pigment heretofore known only from a laboratory synthesis, is actually a functional cone pigment in animals having the Vitamin A₂ visual system.

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COMMENTS AND COMMUNICATIONS

Comments relating to articles which have recently appeared in the Journal of Cellular and Comparative Physiology and brief descriptions of important observations will be published promptly in this Section. Preliminary announcements of material which will be presented later in more extensive form are not desired. Communications should not in general exceed 700 words.

THE EFFECT OF CARBON DIOXIDE ON HUMAN RED CELLS IN VITRO

E. A. BROWN¹

*Department of Physiology, Medical School, University
of Birmingham, England*

It has been shown (Ponder and Furchgott, '40) that human erythrocytes may change their shape when placed between a glass slide and coverslip. An analysis of this phenomenon, alternative to that given by these workers has been provided by the present writer (Brown, '56). In the course of this work, shape changes were observed which may be of interest.

Many observations were made on washed erythrocytes in 0.9% A.R. sodium chloride solution, in hanging-drop preparations. The drop of cell suspension was placed on a thoroughly washed glass coverslip, which was then supported on a thin plastic slide in which a well had been cut. During many of these observations it was noticed that rhythmic shape changes, from crenated sphere to disc and back again, occurred in the cells under observation. This was due to the observer's breath. By varying the composition of the air in the well of the chamber, it was easily shown that the shape of the cells depended on the CO₂ tension over the drop. A similar situation had been encountered by Furchgott ('40) who had ascribed the shape changes that he saw to a lowering of the pH around the cells by the formation of carbonic acid. Furchgott however considered his cells to be free of the "anti-sphering factor" referred to in the papers so far mentioned, and in the work reported here that would not have been the case, even if the hypothesis of the action of the "anti-sphering factor" advanced by Ponder and Furchgott is accepted. Never-

¹ Present address: Fountain Hospital, Tooting Grove, London, S.W. 17.

theless, lowering of the pH seemed to be the obvious explanation, and to check this, the observations were repeated on cells suspended in isotonic salt solutions buffered by M/15 mixed phosphate solutions at several pH values. The shape changes were not thus abolished, but reversed in direction. Thus, raising the CO₂ tension slightly now caused discoid cells to become spheres or crenated spheres, and lowering it to the original value restored the discoid shape.

Cells depleted of chloride by several washings in pure M/15 phosphate buffer did not show any response to varying CO₂ tension. Addition of some saline restored their activity. Cells which had been treated with 1.2×10^{-2} M sulphamylamide — which inhibits the action of carbonic anhydrase (Keilin and Mann, '41) — showed only very feeble activity; and cells depleted of chloride and suspended in isotonic glucose failed to respond to much larger changes in CO₂ tension than would normally produce a shape change.

Cine-films of these phenomena have been made, and shown at a meeting of the British Physiological Society. The diameters of cells in various phases of the shape changes has been measured in stills from this film, and calculation of the cell volumes made. Whilst not pretending to high accuracy, these measurements do not indicate any volume change to be the cause of the shape changes, which are very rapid. It is therefore postulated that the shape change may be due to a re-orientation of protein constituents of the cell membrane — since the surface area of the cell may change by about 30% as the cell goes from biconcave disc to sphere and vice-versa. Further work to provide a more complete explanation of these shape changes is in progress.

I wish to thank Prof. M. H. Jacobs for his interest in this work.

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THE COUPLING OF POTASSIUM TRANSPORT WITH METABOLISM IN DUCK RED CELLS

I. THE EFFECT OF SODIUM FLUORIDE AND OTHER METABOLIC INHIBITORS

D. C. TOSTESON¹ AND J. JOHNSON²

*Laboratory of Kidney and Electrolyte Metabolism, National Heart Institute,
National Institutes of Health, Public Health Service, Department of Health
Education and Welfare, Bethesda, Maryland*

INTRODUCTION

Duck red cells probably maintain a large electrochemical potential gradient for potassium ions between their cytoplasm and the blood plasma (Tosteson and Robertson, '55). This process requires work, energy for which presumably derives from organic reactions occurring within the cells. The present paper reports experiments which represent an attempt to define the coupling of K transport with metabolism in duck red cells.

The steady state rate of transport of K across the duck red cell surface is faster for cells incubated in nitrogen rather than oxygen. Furthermore, intracellular K is kinetically homogeneous for cells in nitrogen, but heterogeneous for cells in oxygen. Finally, cells in nitrogen degrade glucose by glycolysis, whereas those in oxygen respire. The reactions involved in glycolysis are fewer and better understood than are the many reactions of respiration. For these reasons, duck red cells incubated in nitrogen were chosen as a suitable

¹ Present address: Fellow of the National Foundation for Infantile Paralysis, Department Biological Isotope Research, Zoophysiological Laboratory, Copenhagen, Denmark.

² One of us (J. Johnson) has submitted part of this work as partial fulfillment of the requirements for a Masters's Degree in the Department of Chemistry, Georgetown University.

system for further study of the coupling of K transport with metabolism.

This paper reports the effects of sodium fluoride and other metabolic inhibitors on glycolysis and K transport in fresh duck red cells incubated in nitrogen. The results indicate that K fluxes are poorly correlated with the overall rate of glycolysis in this system. These data will be discussed with particular reference to possible coupling of K transport with specific reactions in the glycolytic sequence.

METHODS

The procedure followed was essentially that described in the previous paper (Tosteson and Robertson, '55). Blood was drawn under sterile conditions from the jugular vein of white male adult Pekin ducks. The blood was centrifuged at $3000 \times G$ for 5 min., the plasma and buffy coat removed and discarded and the unwashed cells resuspended in a bicarbonate buffer of approximately the same composition as protein free plasma. The inhibitor under study was also present in this medium. Inhibitor-free control flasks were included in each experiment. The resultant approximately 20% cell suspension was equilibrated with 95% N_2 : 5% CO_2 at $37^\circ C$. for one-half to one hour, at which time a tracer amount of $K^{42}Cl$ was added to the flask. Aliquots of whole cell suspension, cells and medium removed at intervals (usually 30 and 60 minutes) were treated as previously described (Tosteson and Robertson, '55). In all experiments aliquots of the whole cell suspension taken immediately after addition of K^{42} , and again 3 hours later were analyzed for glucose and lactate. Glucose was determined (with identical results) by the methods of Miller and Van Slyke ('36) and Nelson ('44), while lactate was measured by the method of Barker and Summerson ('41).

From the measurements of K^{42} and total K in cells, medium and whole cell suspension, the K influx was calculated as before (Tosteson and Robertson, '55). Since in all experiments tracer K was placed initially in the medium, the measurement of K influx is more reliable than the measure-

ment of K outflux. Rough measurements of K outflux were also available from the data and will be mentioned when pertinent. Since in most of the experiments on fresh cells, cell K remained approximately in the steady state during the experimental procedure, i.e. outflux approximately equalled influx, the K flux was calculated by the steady state procedure (Tosteson and Robertson, '55). In general, flux values reported in the table are the means of values calculated from each of the experimental points. In any one experiment, flux values calculated from samples taken at different times agreed closely.

RESULTS

1. *Effect of fluoride*

10^{-2}M/l NaF had little effect on glucose consumption, inhibited lactate production moderately and doubled K influx (table 1). $3 \times 10^{-2}\text{M/l}$ NaF inhibited glucose consumption and lactate production, and quadrupled K influx. Cell K remained approximately in the steady state in these experiments so that K outflux must have been comparably increased. It should be noted that cell K was completely exchangeable, and usually kinetically homogeneous (exchange curve single exponential) in the presence of NaF. The stimulation of K transport by NaF probably cannot be attributed to increased free diffusion of the ion since this would have led to rapid net loss of K from the cells. Since Maizels found that plasma protected against the action of NaF on net K and Na transport in chicken red cells (Maizels, '54), we studied the effect of NaF on K transport in duck cells in the presence and absence of plasma. Identical results were obtained in each case. There was a substantial increase in cell Na with both concentrations of NaF studied. This effect occurred to the same extent no matter whether the NaF replaced an equimolar amount of NaCl or was added in addition to the other solutes in the medium. Therefore, it was not due to an increase in the concentration of Na in the medium. Cell water content was

TABLE 1

The effect of fluoride, iodoacetate, and N-ethyl-maleimide on potassium transport in duck red cells

| INHIBITOR | GLUCOSE CONSUMPTION | LACTATE PRODUCTION | H ₂ O _e | (K) _e | (Na) _e | (K) _m | K INFUX |
|------------------------------|-----------------------------------|-----------------------------------|-------------------------------|----------------------|----------------------|-----------------------------------|---------|
| | $\frac{mM}{(LEBC.)} \times (hr.)$ | $\frac{mM}{(LEBC.)} \times (hr.)$ | gm/gm | $\frac{mM}{(LEBC.)}$ | $\frac{mM}{(LEBC.)}$ | $\frac{mM}{(LEBC.)} \times (hr.)$ | |
| None | 2.0 | 4.8 | .675 | 102 | 5.51 | 4.58 | 36 |
| 10 ⁻² M/l NaF | 1.3 | 1.5 | .671 | 98.2 | 18.0 | 6.43 | 70 |
| None | 1.5 | 1.8 | .655 | 103 | 6.82 | 5.40 | 18 |
| 3 × 10 ⁻² M/l NaF | 0.9 | 0.4 | .664 | 94.6 | 20.2 | 6.31 | 66 |
| None | 2.0 | 2.6 | .674 | 102 | 5.51 | 4.58 | 36 |
| 10 ⁻³ M/l IAA | 1.0 | 0.1 | .664 | 98.6 | 9.90 | 7.00 | 18 |
| None | 3.6 | 4.5 | .653 | 104 | 6.92 | 4.98 | 19 |
| 10 ⁻³ M/l IAA | 0.0 | 0.7 | .636 | 92.4 | 13.0 | 10.1 | 11 |
| None | 2.0 | 2.8 | .655 | 104 | 8.31 | 5.28 | 23 |
| 10 ⁻³ M/l NEM | 0.2 | 1.2 | .656 | 102 | 7.44 | 5.93 | 23 |
| 10 ⁻³ M/l NEM | 0.0 | 0.0 | .610 | 90.2 | 6.77 | 10.5 | 21 |

The data in the table were obtained from experiments on fresh diluted duck blood incubated at 37°C. for several hours in 95% N₂:5% CO₂ with glucose as substrate. The mean values shown were derived from 3 experiments in 10⁻² M/l NaF, 8 experiments in 3 × 10⁻² M/l NaF, 3 experiments in 10⁻³ M/l iodoacetate (IAA), 3 experiments with 10⁻² M/l IAA and 1 experiment each in 10⁻³ and 10⁻² M/l N-ethyl-maleimide (NEM). Mean values from simultaneously run controls are shown in each case.

not affected by 10^{-2}M/l NaF and decreased only slightly in $3 \times 10^{-3}\text{M/l NaF}$.

Thus, the most impressive effect of NaF on this system is the uncoupling of K transport from glycolysis. However, this process occurs in the opposite sense to the uncoupling of phosphorylation from oxidation observed in the presence of 2,4 dinitrophenol (DNP). In that instance, the energy source (oxidation of substrate) is stimulated while the utilization of energy (esterification of inorganic phosphate) is inhibited. In the present case, the energy source (glycolysis) is inhibited while the utilization of energy for K transport *apparently* proceeds at an accelerated rate. This matter will be considered in greater detail in the discussion. Suffice it to say here that it appears necessary to posit that NaF stimulates some such process as exchange diffusion (Ussing, '54).

2. *Effect of iodoacetate and N-ethyl maleimide*

10^{-3}M/l iodoacetate (IAA) inhibited glucose consumption moderately, lactate production markedly, and K influx moderately (table 1). 10^{-2}M/l IAA reduced glucose consumption and lactate production markedly and K influx moderately. Some net K loss occurred in these experiments so that K outflux was not reduced as much as K influx. Cell water content was reduced slightly in 10^{-3}M/l , and substantially in 10^{-2}M/l IAA. Thus, in contrast to NaF, IAA does not uncouple glycolysis from K influx but rather inhibits both processes.

10^{-3}M/l N-ethyl maleimide (NEM) inhibited both glucose consumption and lactate production but had little effect on K influx (table 1). 10^{-2}M/l NEM had a similar effect. The failure to observe inhibition of K influx in 10^{-2}M/l NEM may have been due to the opening of pathways for K diffusion since there was substantial net loss of K from the cells in these experiments, i.e. K outflux was increased. The dramatic fall in cell water content and the absence of increase in cell sodium in 10^{-2}M/l NEM suggest that, if cation diffusion is increased

under these conditions, the effect is quite selective and accelerates K but not Na diffusion. This type of effect has been observed with NaF in human red cells (Wilbrandt, '40).

3. Effect of arsenate

10^{-2} M/l arsenate stimulated glucose consumption, lactate production and K influx (table 2). These experiments were done in the absence of inorganic phosphate. Removal of inorganic phosphate without addition of arsenate had no effect on glycolysis or K transport. Thus, arsenate, like NaF, stimulates K influx, but in contrast with NaF, stimulates glycolysis.

4. Effect of 2,4 dinitrophenol

10^{-3} M/l 2,4 dinitrophenol (DNP) slightly increased glucose consumption and lactate production but slightly decreased K influx (table 2). 10^{-2} M/l DNP depressed both glucose consumption moderately and lactate production slightly, but inhibited K influx markedly. Thus, DNP produced a type of uncoupling of K influx from glycolysis which is similar to the effect of this compound oxidative phosphorylation i.e. energy release is stimulated or relatively unimpaired but energy utilization markedly inhibited. Clearly, the type of uncoupling of K influx from glycolysis produced by DNP is opposite to that produced by NaF.

5. Effect of other inhibitors

A number of other compounds known to affect one or more enzymes presumed to be present in duck red cells did not affect K transport in duck red cells incubated in nitrogen (table 3). These included azide, nicotinamide, diamox (2 acetylamine - 1,3,4 - thiadiazole - 5 sulfonamide) and cyanide. One aspect of the cyanide effect is worthy of note. Cyanide converted the metabolism of duck red cells in O_2 from respiration to glycolysis. This did not produce acceleration of

| INHIBITOR | GLUCOSE CONSUMPTION $mM/(l.RBC.) \times (hr.)$ | LACTATE PRODUCTION $mM/(l.RBC.) \times (hr.)$ | H ₂ O ₂ gm/gm | (K) _c | (Na) _c | (K) _m | mM/l | $mM/(l.RBC.) \times (hr.)$ |
|-------------------------------|--|---|--|------------------|-------------------|------------------|--------|----------------------------|
| None | 1.9 | 3.8 | .656 | 105 | 7.68 | 5.88 | 29 | |
| 10 ⁻² M/l Arsenate | 5.2 | 5.8 | .652 | 106 | 9.68 | 6.12 | 42 | |
| None | 1.0 | 1.9 | .655 | 103 | 6.38 | 5.76 | 34 | |
| 10 ⁻⁵ M/l DNP | 1.8 | 2.4 | .656 | 102 | 6.70 | 6.06 | 36 | |
| None | 2.2 | 3.6 | .652 | 102 | 7.84 | 5.69 | 30 | |
| 10 ⁻⁴ M/l DNP | 2.9 | 2.8 | .668 | 96.5 | 11.3 | 6.74 | 34 | |
| None | 2.0 | 3.4 | .653 | 102 | 7.41 | 5.67 | 30 | |
| 10 ⁻³ M/l DNP | 2.4 | 3.8 | .657 | 98.2 | 9.94 | 7.34 | 25 | |
| None | 1.6 | 3.0 | .656 | 102 | 6.57 | 5.62 | 31 | |
| 10 ⁻² M/l DNP | 0.8 | 2.7 | .636 | 99.0 | 8.40 | 9.18 | 11 | |

The data in the table were obtained during experiments in which fresh diluted duck blood was incubated at 37°C. in 95% N₂:5% CO₂ with glucose as substrate. The mean values shown were calculated from 2 experiments in 10⁻² M/l arsenate, 1 experiment in 10⁻³ M/l 2,4 dinitrophenol (DNP), 2 experiments in 10⁻⁴ M/l DNP, 3 experiments in 10⁻⁵ M/l DNP and 1 experiment in 10⁻² M/l DNP. Mean values calculated from simultaneously run control experiments are shown in each case.

TABLE 3
Effect of several inhibitors on K transport

| INHIBITOR | GLUCOSE CONSUMPTION $mM/(l.RBC.) \times (hr.)$ | LACTATE PRODUCTION $mM/(l.RBC.) \times (hr.)$ | H ₂ O ₂ gm/gm | (K) _c | (Na) _c | (K) _m | $mM/(l.RBC.) \times (hr.)$ |
|---|--|---|--|------------------|-------------------|------------------|----------------------------|
| None | 2.2 | 4.5 | .655 | 108 | 8.80 | 6.14 | 27 |
| 10 ⁻³ M/l Azide | 1.5 | 4.4 | .645 | 106 | 6.20 | 6.25 | 28 |
| 10 ⁻² M/l Azide | 3.4 | 2.5 | .651 | 102 | 10.1 | 7.03 | 27 |
| None | | | 6.28 | 108 | 10.0 | 12.8 | 22 |
| 10 ⁻³ M/l Diamox | | | 6.42 | 111 | 9.28 | 9.41 | 22 |
| None | | | .689 | 103 | 6.92 | 5.12 | 14 |
| 5 × 10 ⁻³ M/l Nicotinamide | | | | 101 | | 8.35 | 13 |
| 10 ⁻³ M/l Cyanide-N ₂ | 3.0 | 8.1 | .650 | 108 | 20.1 | 5.64 | 17 |
| 10 ⁻³ M/l Cyanide-O ₂ | 3.2 | 7.4 | .608 | 97.1 | 11.4 | 6.70 | 7.5 |

The above data were obtained on fresh diluted duck blood incubated at 37°C. with glucose as substrate in 95% N₂:5% CO₂ except for one set of cyanide experiments done in 95% O₂:5% CO₂. The mean values in the table represent the results of one experiment each in 10⁻³ M/l azide, 10⁻² M/l azide, and 5 × 10⁻² M/l nicotinamide. Two experiments were done in 10⁻² M/l diamox, and 10⁻³ M/l cyanide in N₂ and O₂. In all instances save with cyanide, simultaneously obtained control values are included.

TABLE 4
Effect of several inhibitors combined with fluoride on K transport

| INHIBITOR | GLUCOSE CONSUMPTION $mM/(lRBC.)$ $\times (hr.)$ | LACTATE PRODUCTION $mM/(lRBC.)$ $\times (hr.)$ | H ₂ O _e gm/gm | (K) _e | (Na) _e | (K) _m | K INFUX $mM/(lRBC.)$ $\times (hr.)$ |
|-------------------------------------|--|---|--|------------------|-------------------|------------------|---|
| None | 1.0 | 1.0 | .654 | 104 | 6.98 | 6.24 | 24 |
| 3 $\times 10^{-2}$ M/1 NaF | 0.8 | 0.4 | .665 | 96.4 | 14.3 | 5.88 | 24 |
| NaF + 10 ⁻³ M/1 IAA | 1.0 | 0.2 | .668 | 97.3 | 14.6 | 6.29 | 80 |
| None | 1.0 | 1.0 | .654 | 104 | 6.98 | 6.24 | 24 |
| 3 $\times 10^{-2}$ M/1 NaF | 0.8 | 0.4 | .665 | 96.4 | 14.3 | 5.88 | 90 |
| NaF + 10 ⁻² M/1 DNP | 0.2 | 0.2 | .662 | 96.1 | 13.9 | 6.95 | 14 |
| None | 0.9 | 0.9 | .655 | 105 | 7.04 | 5.72 | 22 |
| 3 $\times 10^{-2}$ M/1 NaF | 0.9 | 0.5 | .670 | 96.7 | 16.7 | 5.60 | 86 |
| NaF + 10 ⁻² M/1 Arsenate | 0.1 | 0.4 | .646 | 100 | 18.1 | 7.45 | 77 |

The above data were obtained on fresh diluted duck blood incubated at 37°C. in 95% N₂:5% CO₂ with glucose as substrate. Two experiments were done in NaF + IAA, two in NaF + DNP, and four in NaF + arsenate. Mean values obtained from simultaneously run flasks containing either NaF alone or no inhibitor are also shown.

TABLE 5
Effect of several inhibitors combined with iodoacetate on K transport

| INHIBITOR | GLUCOSE CONSUMPTION $mM/(lRBC.)$ $\times (hr.)$ | LACTATE PRODUCTION $mM/(lRBC.)$ $\times (hr.)$ | H ₂ O _e gm/gm | (K) _e | (Na) _e | (K) _m | K INFUX $mM/(lRBC.)$ $\times (hr.)$ |
|-------------------------------------|--|---|--|------------------|-------------------|------------------|---|
| None | 3.1 | 4.1 | .655 | 102 | 7.08 | 5.17 | 23 |
| 10 ⁻² M/1 IAA | 0.0 | 0.0 | .633 | 95.0 | 10.7 | 10.7 | 13 |
| IAA + 10 ⁻³ M/1 DNP | 0.0 | 0.0 | .631 | 94.8 | 9.60 | 10.1 | 6.0 |
| None | 3.1 | 4.2 | .655 | 104 | 7.17 | 5.24 | 22 |
| 10 ⁻² M/1 IAA | 0.0 | 0.0 | .633 | 95.0 | 10.7 | 10.7 | 13 |
| IAA + 10 ⁻³ M/1 Arsenate | 0.0 | 0.1 | .623 | 98.6 | 11.8 | 9.04 | 14 |

The above data were obtained on fresh diluted duck blood incubated at 37°C. in 95% N₂:5% CO₂ with glucose as substrate. One experiment each in combined IAA-DNP and combined IAA-arsenate are included. Mean values from simultaneously run flasks containing either IAA alone or no inhibitor are also shown.

K transport as is the case when respiration is converted to glycolysis by replacement of O_2 with N_2 .

6. *Effect of combined inhibitors*

Several experiments were done with both $3 \times 10^{-2}M/l$ NaF and one other inhibitor present in the incubation medium (table 4). When fluoride and $10^{-3}M/l$ IAA were both present simultaneously the fluoride effect predominated and K transport was accelerated. Similar results were obtained with NaF and $10^{-2}M/l$ IAA. In other words, NaF reversed the inhibition of K transport produced by IAA. NaF had little effect on the inhibition of K influx produced by $10^{-2}M/l$ DNP. When NaF was present with $10^{-2}M/l$ arsenate glycolysis was inhibited but K influx was stimulated as compared with the values observed in arsenate alone.

Experiments were also done with $10^{-2}M/l$ IAA in combination with other inhibitors (table 5). IAA combined with $10^{-2}M/l$ arsenate resulted in marked inhibition of glycolysis and K influx. Similar results were obtained with IAA plus $10^{-3}M/l$ DNP.

DISCUSSION

The effects of the various metabolic inhibitors on K transport in fresh duck red cells were obviously complicated. The action of the compounds on ion transport was poorly correlated with their effect on the overall rate of glycolysis. Thus, K influx was both decreased (IAA) and increased (NaF) in the presence of compounds which inhibited glycolysis. Conversely, K transport was both slowed (DNP) and accelerated (arsenate) in the presence of substances which stimulated glycolysis. These facts suggest that K transport is quite independent of the rate of energy release from the reactions of glycolysis. It should be emphasized that the poor correlation between K fluxes and glycolysis noted in these experiments is not a general property of K transport in duck red cells. A companion paper (Tosteson and Johnson, '57) reports

the close relation between the two processes observed in duck red cells which have been stored for 24 hours at 37°C.

In a previous communication (Tosteson and Robertson, '55), evidence was adduced to support the conclusion that an appreciable fraction of K transport in duck red cells occurs by some such process as exchange diffusion (Ussing, '54). The data on K transport in the presence of 3×10^{-2} M/1 NaF strengthen the energetic argument which supports this conclusion. The assumptions underlying this argument are as follows. (1) The activity coefficient of K is the same inside and outside the cells. (2) The electrical potential difference across the red cell membrane may be estimated from the ratio of chloride concentration in cells and medium. (3) K influx is a thermodynamically reversible process. (4) None of energy from K outflux is available for K influx. From these assumptions, it can be calculated that about 110 cal./(1 RBC) \times (hr.) would be required to maintain K influx at the value observed in the presence of 3×10^{-2} MNaF³. This value exceeds the energy released during glycolysis, a maximum of about 40 cal./(1 RBC) \times (hr.) in these experiments. It is likely that assumption (4) above is wrong and that some of the energy from outflux of K is made available for influx.

This conclusion derives further support from a consideration of the stoichiometry of the situation. If one assumes that 95% of the inward K transport occurs in 1:1 combination with a carrier molecule which is synthesized on the outer cell surface and destroyed on the inside of the plasma membrane, 63 mM of such a compound must be made and consumed /(1RBC) \times (hr.) to account for K influx in duck red cells in NaF. Under these circumstances, 0.4 mM of lactate/(1 RBC.) \times (hr.) are formed [or, assuming the normal ratios for glycolysis, 0.4 mM phosphate esterified and diphosphopyridine nucleotide (DPN) reduced and reoxidized]. Several explanations for this discrepancy are possible.

³In a previous paper (Tosteson and Robertson, '55), an erroneously low figure was given for the energy required for K transport in the presence of NaF. The correct figure is that cited above, not 78 cal./(1RBC.) \times (hr.) as mentioned in the earlier article.

(1) There is some organic compound which turns over during glycolysis at 100 times the rate of DPN. This seems unlikely. (2) More than one K ion combines with the organic carrier molecule. (3) It is not necessary that the organic molecule be synthesized and degraded in order to effect the exchange of K between medium and cytoplasm, i.e. the molecular site for K outflux is also available for influx and exchange diffusion occurs. Of course, a combination of these alternatives is possible.

Finally, it is noteworthy that most of the inhibitors studied (NaF, DNP, arsenate) affected K outflux *pari passu* with K influx. Probably these compounds act on K transport mainly by altering exchange diffusion of the ion, rather than by disturbing a tight coupling between K influx by active transport and glycolysis. At present, it is not possible to quantitatively distinguish between exchange diffusion and active transport of K in duck red cells. However, it is probable that the latter, as well as the former, mechanism of transport is operative since duck red cells can accumulate net K under certain conditions (Tosteson and Johnson, '57). We will now examine the data reported above for clues to the molecular site of K exchange diffusion (and active transport) in this system.

It is attractive to speculate that the various inhibitors affect K transport by altering the concentration and/or turnover rate of one or more phosphate esters. IAA probably blocks glycolysis by inhibiting 3-phosphoglyceraldehyde-dehydrogenase (Green, Needham and Dewan, '37), the enzyme which catalyzes the only reaction in the Meyerhof-Embden scheme in which esterification of inorganic phosphate occurs. Reduced synthesis probably leads to a fall in concentration and/or turnover rate of phosphate esters under these circumstances. We have observed a reduction in K influx in the presence of IAA. On the other hand, NaF probably blocks glycolysis by inhibiting enolase (Lohmann and Meyerhof, '34), a step which follows the primary esterification of inorganic phosphate. Furthermore, the compound inhibits the activity

of phosphatases (Roche, '50), adenylate kinase (Siekevitz, and Potter, '53; Barkulis and Lehninger, '51) and certain (Ochoa '41, '43; Judah and Williams-Ashman, '51; Novikoff, Hecht, Podber and Ryan, '52) but not all (Lardy and Wellman, '53), adenosine triphosphatases (ATPases). Therefore, the concentration of certain phosphate esters may be expected to remain high or even increase, as has been observed of di-phosphoglyceric acid in human red cells exposed to NaF (Mueller and Hastings, '51). We have observed that K fluxes are increased in the presence of NaF. Similarly, DNP stimulates ATPase activity and might be expected to reduce the concentration of one or more phosphate esters (Lardy and Wellman, '53). K fluxes are reduced in cells exposed to DNP. In the case of arsenate, however, the argument breaks down. This compound probably acts on glycolysis by competing with phosphate for the acyl-enzyme complex between 3-phosphoglycerate and 3-phosphoglyceraldehyde-dehydrogenase (Krimsky and Racker, '55). The resultant 1-arseno 3-phosphoglyceric acid is unstable and breaks down to yield 3-phosphoglyceric acid and arsenate. Thus, flow of substrate through the reaction sequence is actually stimulated but primary esterification of inorganic phosphate does not occur. Despite the probable resultant fall in concentrations of phosphate esters, K fluxes are increased in the presence of arsenate.

An alternative interpretation of the inhibitor data involves the enzyme pyruvic phospho-transferase. This enzyme requires K for activity (Kachmar and Boyer, '53), and has been found to be more active in the red cells of chickens than in those of mammals (Solvonuk and Collier, '55). The enzyme is inhibited some 30% by 10^{-3} M/l IAA but not affected by NaF (Solvonuk and Collier, '55). A correlation of these observations with the inhibition of K influx in duck red cells by IAA and actual stimulation by NaF is possible. Thus, we might deduce that formation of ATP by the reaction of phospho-enol-pyruvate with ADP (catalyzed by pyruvic phosphotransferase) was necessary for K transport. Such an hypothesis would account for the effect of arsenate as well as

of IAA and NaF on K transport, since ATP synthesis by this reaction is probably accelerated when glycolysis is stimulated by arsenate. The slight inhibition of K transport by DNP is more difficult to explain in terms of this hypothesis, but could be related to increased ATPase activity.

Finally, a consideration of the organic phosphate composition of avian red cells suggests a more specific and provocative, if more speculative interpretation of the data. Fifteen years ago, Rapoport found that the red cells of several species of birds contain a large quantity of phytic acid (inositol hexa phosphoric acid ester) (Rapoport, '40, '41 and Guest, '41). In view of the large negative charge of this compound, it is interesting to speculate that phytic acid provides a site for K exchange diffusion in duck red cells. Several facts support this interpretation. Thus, NaF, which stimulates K exchange diffusion in duck red cells, inhibits phytase, the enzyme catalyzing the hydrolysis of phytic acid (Rapoport, Leva and Guest, '41a). Prolonged incubation of goose red cells in N₂ in the absence of glucose markedly reduces the concentration and turnover rate of phytic acid (Rapoport, Leva and Guest, '41b), while similar treatment of duck red cells markedly reduces K fluxes (Tosteson and Johnson, '57). Finally, phytic acid is not present in mammalian red cells, in which exchange diffusion does not appear to be an important mechanism of K transport (Tosteson, '55). It would be interesting to know whether K exchange diffusion occurs in all red cells which contain phytic acid.

Clearly no completely adequate explanation of the effects of the inhibitors on K transport is now available. However, the pattern of action of these compounds on K transport may provide a map with which to localize the organic reactions coupled with ion transport.

In a previous paper (Tosteson and Robertson, '55), it was suggested that the stimulation of K transport in duck red cells by hypoxia might be due to the conversion of metabolism from respiration to glycolysis under these circumstances. The effect of cyanide reported above appears to rule out this

possibility. The stimulation of K transport by hypoxia persisted in the presence of cyanide when both the N_2 and the O_2 system were degrading glucose by glycolysis. The explanation for the effect of O_2 on K transport must be sought elsewhere.

SUMMARY

The effects of various metabolic inhibitors on K influx into fresh duck red cells incubated at 37° in 95% N_2 :5% CO_2 with glucose as substrate were studied. $10^{-2}M/l$ IAA inhibited, while $3 \times 10^{-2}M/l$ NaF stimulated K influxes although both compounds inhibited glycolysis. $10^{-3}M/l$ DNP slightly inhibited, while $10^{-2}M/l$ arsenate stimulated K fluxes, although both compounds stimulated glycolysis. $10^{-2}M/l$ NEM did not affect K influx but stimulated K outflux. Azide, cyanide, diamox and nicotinamide did not affect K transport.

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THE COUPLING OF POTASSIUM TRANSPORT WITH METABOLISM IN DUCK RED CELLS

II. THE EFFECT OF ADENOSINE AND OTHER SUBSTRATES

D. C. TOSTESON¹ AND J. JOHNSON

*Laboratory of Kidney and Electrolyte Metabolism, National Heart Institute,
Bethesda, 14, Maryland*

ONE FIGURE

INTRODUCTION

Potassium transport in duck cells incubated in nitrogen apparently requires a constant supply of energy from the reactions of glycolysis (Tosteson and Robertson, '56; Tosteson and Johnson, '57). We have previously reported experiments which were designed to characterize this coupling of K transport with glycolysis by the use of metabolic inhibitors (Tosteson and Johnson, '57). This paper reports the effect of various substrates on K transport in this system.

Shortly after this study was begun, it was discovered that removal of glucose produced no immediate effect on K transport in duck red cells incubated in nitrogen. However, prolonged incubation of such a system (free of exogenous substrate) resulted in a reduction of both K influx and outflux to about 5% of their initial values. Addition of glucose to this stored system neither regenerated K transport nor produced glycolysis. However, addition of glucose plus adenosine produced both regeneration of K transport and glycolysis. These and other observations on the regeneration of K transport in stored duck red cells are described in this report.

¹ Present address: Fellow of the National Foundation for Infantile Paralysis, Dept. of Biological Isotope Research, Zoophysiological Laboratory, Copenhagen, Denmark.

METHODS

Unwashed sterile duck red cells were obtained as before and suspended in medium which contained no glucose. The resultant approximately 10% cell suspension contained 100,000 units of penicillin and, in later experiments, also 0.5 gm. of streptomycin per 150 ml. Control experiments indicated that the antibiotics did not affect K transport in the system. The cell suspension was equilibrated with 95% N₂ : 5% CO₂ and allowed to incubate for 16–24 hours at 37° C. The suspension was then centrifuged under oil and the cells resuspended in medium containing glucose and/or other substrates, and a tracer amount of K⁴² Cl. This new approximately 20% cell suspension was then re-incubated in 95% N₂ : 5% CO₂ at 37° C. Aliquots of whole cell suspension, cells and medium were taken immediately after addition of the cells to the fresh medium, one and two hours later and were treated as before (Tosteson and Johnson, '57). From the data, K influx was calculated by the unsteady state procedure (Tosteson and Johnson, '57).

In those experiments in which the effect of various nucleosides and purines was studied, 10% trichloroacetic acid filtrates were made of the medium before the addition of the cells, and of medium and whole cell suspension immediately after the addition of cells, and 2–3 hours later. One aliquot of each of these filtrates was diluted with buffer to pH 7 and the concentration of nucleoside or purine estimated by measuring the optical density at the appropriate wave length in the ultra violet (Kalekar, '47 a, b). Measurements of inorganic phosphate (Fiske and SubbaRow, '25) were also made on these trichloroacetic acid filtrates.

RESULTS

1. Effect of time on K transport

When duck red blood cells were allowed to incubate at 37° C. in 95% N₂ : 5% CO₂ in the absence of substrate, K influx fell progressively during the incubation period (table 1). After 24 hours storage under these conditions K influx

| INCUBATION TIME | GAS PHASE | (GLUCOSE) | H ₂ O _c | (K) _c | (Na) _c | (K) _m | K INFUX |
|--------------------|------------------------------------|-----------|-------------------------------|------------------|-------------------|------------------|-----------------------------|
| hrs | % | mM/l | gm/gm | mM/l RBC | | mM/l | mM/(l RBC) <i>x</i> (hr) |
| 1 | 95N ₂ :5CO ₂ | 20 | .626 | 114 | 6.3 | 4.84 | 21 |
| 1 | 95N ₂ :5CO ₂ | 0 | .659 | 104 | 12.2 | 5.40 | 16 |
| 9 | 95N ₂ :5CO ₂ | 0 | .660 | 94.9 | 18.9 | 6.60 | 1.2 |
| 20-23 | 95N ₂ :5CO ₂ | 0 | .664 | 76.4 | 37.1 | 17.1 | 0.3 |
| 20-23 | 95N ₂ :5CO ₂ | 20 | .652 | 88.2 | 22.4 | 25.2 | 9.3 |
| 23 | 95O ₂ :5CO ₂ | 0 | .600 | 81.9 | 26.6 | 30.4 | 7.4 |
| 23 | 95O ₂ :5CO ₂ | 20 | .586 | 99.4 | 15.4 | 25.9 | 17 |

The above data were obtained from diluted duck blood incubated for various lengths of time at 37°C. in 95% N₂ : 5% CO₂ or 95% O₂ : 5% CO₂ with or without glucose. The mean values in the table were derived from 1 experiment of 1 hr. incubation with glucose in N₂, 2 experiments of 1 hr. incubation without glucose in N₂, 1 experiment of 9 hr. incubation without glucose in N₂, 3 experiments of 20-23 hr. incubation without glucose in N₂, 1 experiment of 20-23 hr. incubation with glucose in N₂, and 1 experiment each of 23 hr. incubation in O₂ with and without glucose.

TABLE 2

Regeneration of K transport in stored cells

| GAS PHASE | GLUCOSE | (ADENOSINE) | GLUCOSE CONSUMPTION | LACTATE PRODUCTION | H ₂ O _c | (K) _c | (Na) _c | (K) _m | K INFUX |
|------------------------------------|---------|-------------|------------------------|-----------------------|-------------------------------|------------------|-------------------|------------------|---------|
| % | mM/l | mM/l | mM/(l RBC) × (hr) | | gm/gm | mM/(l RBC) | mM/l | mM/(l RBC) | × (hr) |
| 95N ₂ :5CO ₂ | 5 | 0 | 0.0 | 0.4 | .671 | 78.0 | 29.9 | 8.36 | 0.9 |
| 95N ₂ :5CO ₂ | 0 | 10 | — | 0.1 | .673 | 74.9 | 30.8 | 9.95 | 1.3 |
| 95N ₂ :5CO ₂ | 5 | 10 | 2.8 | 4.8 | .671 | 81.4 | 23.6 | 8.61 | 6.0 |

The data in the above table were obtained on duck cells which had been stored overnight at 37°C. in 95% N₂ : 5% CO₂ in the absence of glucose. The mean values in the table represent measurements made during incubation of these stored cells at 37°C. under the conditions noted. The concentrations of K, Na and H₂O in cells and medium are those observed at the end of this 2 hr. incubation. There were 7 experiments on cells incubated with glucose but without adenosine, 5 experiments on cells without glucose but with adenosine and 7 experiments on cells with both glucose and adenosine.

had fallen to about 5% of its initial value. This reduction in K influx occurred at a time when the cells still contained much more K than did the medium. Therefore, K outflux must have been comparably reduced. Prolonged incubation of duck red cells in N_2 in the presence of glucose produced only a moderate decline in K influx. This was also true for cells stored in O_2 in the absence of glucose. K influx did not decrease with time in cells incubated for 24 hours in O_2 in the presence of glucose.

2. *Regeneration of K transport in stored cells*

Attempts were made to reverse the markedly slowed K transport in duck cells which had been stored over night at 37° C. in a medium containing no substrate in 95% N_2 : 5% CO_2 . Addition of glucose to the stored cells did not accelerate K influx or produce glycolysis (table 2). Adenosine alone was also without effect. Addition of both of these compounds simultaneously increased K influx about five-fold. Comparison of table 2 with table 1 shows that this value of K influx was about the same as that observed in duck cells after 24 hours incubation in N_2 in the *presence* of glucose. Net accumulation of K and extrusion of Na, as well as brisk glycolysis were also observed upon addition of both glucose and adenosine to cells which had been stored in the absence of glucose (table 2). Because of these results, experiments were done to define the specificity of the nucleoside and the hexose in regeneration of K transport in this stored system.

3. *Specificity of nucleoside effect on K influx*

The data summarized in table 3 indicate that the regenerative effect of adenosine on K influx and glycolysis in duck red cells is quite specific. Of the compounds studied, only adenine plus ribose, and to some extent adenine alone could substitute for adenosine. In all cases, regeneration of K transport was closely correlated with regeneration of glycolysis.

TABLE 3
Effect of nucleosides and related compounds on K transport

| GAS PHASE | (GLUCOSE) | (NUCLEOSIDE) | GLUCOSE CONSUMPTION | LACTATE PRODUCTION | H ₂ O _c | (K) _c | (Na) _c | (K) _m | K INFUX |
|------------------------------------|-----------|-----------------------|------------------------|-----------------------|-------------------------------|------------------|-------------------|------------------|-----------------------|
| % | mM/l | mM/l | mM/ (1 RBC) × (hr) | | gm/gm | mM/ (1 RBC) | | mM/l | mM/ (1 RBC) × (hr) |
| 95N ₂ :5CO ₂ | 5 | 0 | 0.0 | 0.1 | .682 | 75.6 | 33.7 | 8.33 | 0.6 |
| | 5 | Adenosine 10 | 3.1 | 5.0 | .688 | 82.4 | 26.4 | 6.53 | 2.7 |
| | 5 | Inosine 10 | 0.2 | 0.6 | .685 | 78.0 | 31.1 | 7.45 | 0.7 |
| | 5 | Guanosine 10 | 0.0 | 0.3 | .691 | 68.6 | 40.1 | 11.0 | 1.1 |
| 95N ₂ :5CO ₂ | 5 | 0 | 0.0 | 0.3 | .664 | 83.2 | 28.8 | 8.10 | 0.7 |
| | 5 | Adenosine 5 | 1.2 | 3.4 | .669 | 83.8 | 25.1 | 7.91 | 2.7 |
| | 5 | Adenine 5 | 1.0 | 2.0 | .673 | 83.3 | 26.4 | 7.60 | 1.9 |
| | 5 | ATP 5 | 0.3 | 1.2 | .676 | 75.3 | 35.1 | 10.6 | 1.6 |
| 95N ₂ :5CO ₂ | 5 | 0 | 0.0 | 0.2 | .668 | 81.8 | 29.5 | 8.08 | 0.7 |
| | 5 | Adenosine 5 | 1.8 | 3.9 | .667 | 84.6 | 24.1 | 7.85 | 2.8 |
| | 5 | Adenine 5 Ribose 5 | 2.6 | 3.6 | .674 | 84.4 | 23.9 | 7.50 | 3.4 |

The data in the table were obtained on duck red cells which had been stored overnight at 37°C. in 95% N₂: 5% O₂ in the absence of glucose. The mean values in the table represent measurements made during a 2 hr. incubation of these stored cells at 37°C. under the condition noted. The concentrations of K, Na and H₂O in cells and medium were observed at the end of this 2 hr. incubation. There was one experiment each on cells incubated under the following conditions: in the presence of 5 mM/1 adenine plus 5 mM/1 ribose, 10 mM/1 guanosine and 10 mM/1 inosine. There were 2 experiments each in cells incubated in the presence of 5 mM/1 adenine and 5 mM/1 adenosine triphosphate (ATP). Values from simultaneously run control flasks containing either glucose alone or glucose plus adenosine are also shown.

TABLE 4
Effect of various substrates on K transport

| GAS PHASE | (ADENOSINE) | (SUBSTRATE) | LACTATE PRODUCTION | H ₂ O _c | (K) _c | (Na) _c | (K) _m | K INFLUX |
|------------------------------------|-------------|----------------------|-----------------------|-------------------------------|------------------|-------------------|------------------|-----------------------|
| % | mM/l | mM/l | mM/ (1 RBC) x (hr) | gm/gm | mM/l RBC | | mM/l | mM/ (1 RBC) x (hr) |
| 95N ₂ :5CO ₂ | 10 | None | 0.1 | .667 | 74.7 | 28.9 | 8.35 | 1.1 |
| | 10 | Glucose 5 | 4.8 | .663 | 81.6 | 23.4 | 8.45 | 6.3 |
| | 10 | Mannose 5 | 3.8 | .651 | 83.2 | 19.2 | 8.70 | 5.6 |
| | 10 | Galactose 5 | 0.1 | .681 | 75.7 | 31.8 | 7.65 | 1.4 |
| | 10 | Glycerol 5 | 0.7 | .707 | 72.7 | 30.3 | 8.68 | 1.1 |
| 95N ₂ :5CO ₂ | 10 | Glucose 5 | 2.3 | .678 | 80.5 | 25.6 | 7.50 | 3.4 |
| | 10 | Ribose 5 | 0.2 | .679 | 73.7 | 33.4 | 7.90 | 0.9 |
| | 10 | Ethyl Oxaloacetate 5 | 0.1 | .701 | 76.2 | 34.1 | 8.15 | 1.0 |
| 95N ₂ :5CO ₂ | 10 | Pyruvate 5 | 0.2 | .658 | 78.2 | 33.4 | 8.33 | 1.0 |
| | 10 | None | 0.0 | .668 | 74.1 | 33.8 | 8.31 | 1.0 |
| | 10 | Glucose 5 | 4.1 | .676 | 76.1 | 27.8 | 7.47 | 4.7 |
| | 10 | Glycero Phosphate 5 | 0.3 | .657 | 78.3 | 34.9 | 8.25 | 1.1 |
| 95N ₂ :5CO ₂ | 10 | None | 0.5 | .677 | 75.6 | 31.1 | 8.07 | 1.2 |
| | 10 | Glucose 5 | 4.9 | .667 | 77.3 | 25.0 | 7.26 | 5.6 |
| | 10 | Glyceraldehyde 5 | 0.7 | .667 | 75.4 | 31.5 | 8.08 | 1.9 |

The above data were obtained on duck red cells which were stored overnight at 37°C. in the absence of glucose. The values in the table represent measurements made during a 2 hr. incubation of these cells at 37°C. under the conditions noted. The concentrations of K, Na and H₂O in cells and medium are those observed at the end of this 2 hr. incubation. The data represent the results of 3 experiments with glyceraldehyde, 2 with glycerophosphate, and single experiments with each of the other substrates. The values obtained from simultaneously run control flasks containing either adenosine alone or adenosine plus glucose are also shown.

4. *Specificity of hexose on K influx*

The data shown in table 4 indicate that mannose could replace glucose as substrate during the regeneration of K influx and glycolysis in stored duck red cells in the presence of adenosine. All other substances tested were ineffective substitutes for glucose. Attempts to study the coupling of portions of the glycolytic pathway of reactions to K transport by supplying substrates below the level of hexose were unsuccessful. It is not clear whether this result was due to the failure of the substrates to penetrate into the cells or the failure of the compounds to participate in reactions which enter into the glycolysis pathway.

TABLE 5
Adenosine and phosphate in stored cells

| TIME | HCT | (GLUCOSE) _b | (PURINE) _m | (Pi) _m | (Pi) _b | (Pi) _c |
|-----------------------------------|------|------------------------|-----------------------|-------------------|-------------------|-------------------|
| | | mM/l | mM/l | mM/l | mM/l | mM/l |
| Before addition of cells | | | 8.62 | 2.17 | | |
| 5 min. after addition of cells | .202 | 0.00 | 6.55 | 2.14 | 2.92 | 6.00 |
| 2 hrs. after addition of cells | | 0.00 | 4.88 | 2.26 | 3.05 | 6.18 |
| Before addition of cells | | | 8.62 | 2.19 | | |
| 5 min. after addition of cells | .161 | 4.63 | 7.32 | 2.19 | 2.59 | 4.65 |
| 2 hrs. after addition of cells | | 2.78 | 4.56 | 2.21 | 2.14 | 1.80 |

Duck red cells were stored overnight in 95% N₂:5% CO₂ in the absence of glucose at 37° C. The cells were re-incubated in the same gas phase and temperature in a medium containing adenosine in the absence (upper set of figures) and presence (lower set of figures) of glucose. The cell inorganic phosphate concentrations, (Pi)_c, was calculated from measurements of phosphate in the medium, (Pi)_m, and whole cell suspension, (Pi)_b, and the hematocrit. The values in the table are from a single representative experiment. The phosphate results were confirmed in three other experiments. Similar purine results were obtained in four additional experiments with adenosine alone and ten additional experiments with adenosine plus glucose.

5. *Role of adenosine and phosphate in regeneration of K transport in stored cells*

A large amount of adenosine disappears from the medium in the presence or absence of glucose (table 5). The apparent decrease in medium concentration of purine was about 4 mM/l in two hours. Preliminary observations indicate

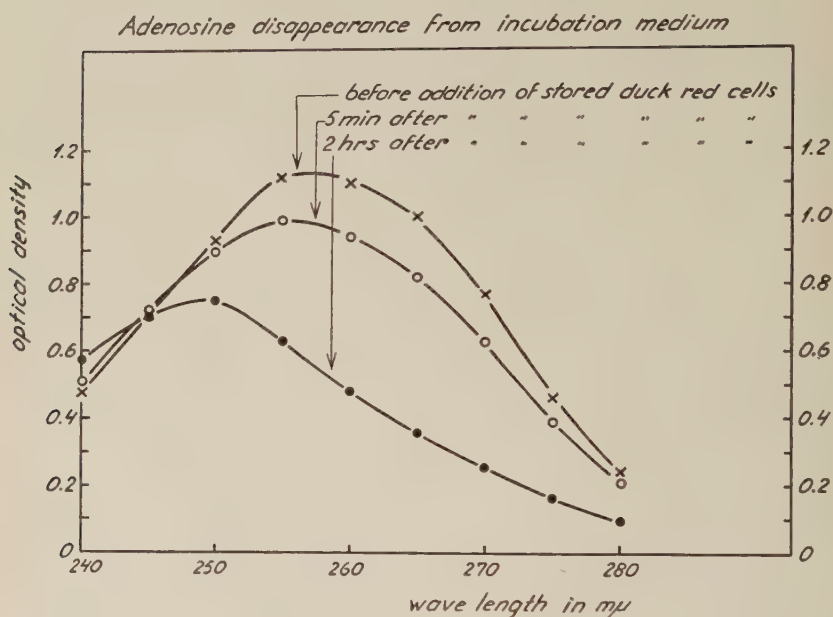


Fig. 1 The above graph shows the effect of addition of duck red cells which had been stored overnight in the absence of glucose on the absorption spectrum of adenosine in the medium. The medium also contained glucose. The optical density is plotted on the ordinate as a function of the wave length in $m\mu$ on the abscissa. The results are from a single experiment which is representative of the three which were done.

that the absorption maximum of the purine shifts from 260 to 250 $m\mu$ on exposure to duck red cells (fig. 1). This spectral shift may be attributable to de-amination of adenosine (or its adenine moiety), since the absorption maximum of inosine (and hypoxanthine) is at 250 $m\mu$ (Kalekar, '47 a). All of the adenosine (or adenine) disappearing from the medium could

be accounted for as inosine (or hypoxanthine). The inosine appears to be evenly distributed between cells and medium. Similar results to those shown in figure 1 were obtained whether or not added glucose was present in the system. The reactions involved in the disappearance of adenosine from the medium were not defined but it is clear that they are not sufficient to regenerate K transport since they occur in the absence of glucose when K influx remains low, as well as in the presence of glucose when K transport is markedly accelerated.

Disappearance of inorganic phosphate from the cells occurred only when K transport and glycolysis were regenerated (table 5). Under conditions in which regeneration did not occur (e.g. with adenosine alone, glucose alone, inosine plus glucose, guanosine plus glucose, adenosine plus glyceraldehyde, adenosine plus glycerophosphate), no net esterification of phosphate was observed.

DISCUSSION

Storage of duck red cells in nitrogen in the absence of glucose results in a dramatic reduction in K fluxes. It is reasonable to conclude that storage inhibits the exchange diffusion which apparently plays a large role in K transport in fresh duck cells (Tosteson and Robertson, '56; Tosteson and Johnson, '57). Several aspects of this problem deserve further investigation. For example, it will be interesting to see if the variation of K influx with K concentration in the medium in adenosine-glucose regenerated stored duck cells confirms a reduction in the exchange diffusion component of K transport (Tosteson and Robertson, '56). Furthermore, a study of the relative concentrations of phytic acid in fresh and stored duck cells would be of interest since this compound may provide a site for K exchange diffusion (Tosteson and Johnson, '57).

The regeneration of K transport in stored duck red cells occurred only when glycolysis was also regenerated. Thus,

despite the failure to isolate specific reactions within the glycolytic sequence which are essential for ion transport, the dependence of K influx on one or more of these reactions was clearly demonstrated. Furthermore, the marked reduction in cell inorganic phosphate which occurs during regeneration is compatible with the view that some phosphate ester necessary for K influx is depleted during storage and resynthesized upon addition of adenosine and glucose.

The regeneration of glycolysis and organic phosphates in stored cells by adenosine is similar to the observations of Gabrio et al. on human red cells (Gabrio, Hennesey, Thomasson and Finch, '55). Significant differences between the two systems exist, however. In human cells under certain conditions the storage lesion is reversed by adenosine alone. In the duck cells glucose must also be present. In human cells inosine and guanosine can replace adenosine but adenine is ineffective (Gabrio et al., '55; Gabrio and Huennekens, '55). In duck cells the opposite obtains. It is not clear whether these discrepancies are referable to differences in the nature of the storage lesion in the two systems or to differences in the regenerating reactions.

The reactions by which adenosine aids in regeneration were not defined. Gabrio et al., ('55) have isolated a rather non-specific nucleoside phosphorylase from human red cells. Since this enzyme catalyzes the phosphorolysis of inosine and guanosine as well as adenosine it probably does not play a specific role in the regeneration of stored duck cells by adenosine. It is, of course, possible that a nucleoside phosphorylase of different specificity is present in duck cells, or that the specificity of adenosine depends on some reaction occurring after phosphorolysis of the nucleoside. However, the regenerative action of adenine raises the possibility that the phosphoribosyl pyrophosphate reaction (Kornberg, '55) might be involved in restoration of glycolysis and K transport in stored duck cells. Also, the fate of the purine leaving the medium and entering the cells remains to be elucidated. As

noted above, conversion to hypoxanthine or a compound containing hypoxanthine is suggested by our preliminary spectral data. It is not clear whether this conversion occurs inside or and outside the cells. These reactions may occur by the same process responsible for hypoxanthine accumulation in stored human blood (Jorgensen and Poulsen, '55). Clearly, much work is necessary before adenosine metabolism in this system is well understood.

TABLE 6
K transport in stored duck red cells

| GAS PHASE | | GLUCOSE CONSUMP- TION | LACTATE PRODUCTION | NET K FLUX |
|------------------------------------|------------------------------------|-----------------------------|-----------------------|--------------------|
| Storage | Incubation | | | |
| % | % | mM/ (1 RBC) × (hr) | mM/ (1 RBC) × (hr) | mM/ (1 RBC) × (hr) |
| 95N ₂ :5CO ₂ | 95N ₂ :5CO ₂ | 0.2 | 0.2 | - 1.1 |
| 95O ₂ :5CO ₂ | 95N ₂ :5CO ₂ | 2.2 | 3.8 | + 5.3 |
| 95O ₂ :5CO ₂ | 95O ₂ :5CO ₂ | 1.0 | 1.6 | + 1.7 |

The above data were obtained on duck red cells which had been incubated at 37°C. for 24 hours in the absence of glucose and K in either 95% N₂:5% CO₂ or 95% O₂:5% CO₂. These stored cells were then re-incubated in a medium containing both glucose and K in either N₂ or O₂. The values in the table were obtained during this re-incubation.

Maizels ('54) has stated that net K re-accumulation in previously cold stored chicken red cells depends on respiration and cannot be driven by glycolysis. In contrast the steady state K flux in fresh duck red cells incubated in N₂ (glycolysis) exceeds the value obtained in O₂ (respiration) (Tosteson and Robertson, '56). The following experiment proves that glycolysis can also support net K accumulation in duck cells. Fresh duck red cells were stored over night at 37° C. in a medium containing neither glucose nor K. Half of the cells were stored in 95% O₂:5% CO₂, the remainder in 95% N₂:5% CO₂. The next day the cells stored in N₂ were incubated at 37° C. in a restoring medium containing 5 mM l glucose and 10 mM l K in N₂. The cells stored in O₂ were incubated in the same restoring medium, but half of the cells

were equilibrated with O_2 , the remainder with N_2 . The results are shown in table 6. Net K re-accumulation failed to occur in cells which were both stored and re-incubated in N_2 . On the other hand, for cells stored in O_2 , net K re-accumulation was actually faster when the restoring gas phase was N_2 (glycolysis) than when it was O_2 (respiration). Clearly, glycolysis can support net K accumulation in cells stored in O_2 . Storage in N_2 at $37^\circ C$. alters the system so that net K accumulation is no longer possible with glucose alone as substrate. Perhaps storage overnight in the absence of O_2 in the cold, as done by Maizels, has a similar effect on K transport in chicken red cells, thus accounting for his failure to observe net K accumulation in the absence of O_2 . A species difference is also possible, of course.

SUMMARY

When duck red cells were stored over night at $37^\circ C$. in 95% N_2 : 5% CO_2 in the absence of glucose, K influx fell to less than 5% of its initial value. Upon re-incubation of these cells in N_2 with glucose alone or adenosine alone, glycolysis failed to occur and K influx remained low. Upon re-incubation in N_2 with glucose and adenosine together, brisk glycolysis occurred and K influx increased five-fold. In the presence of adenosine, mannose but not galactose, ribose, glycerol, phosphoglycerol, glyceraldehyde, ethyloxaloacetate or pyruvate could substitute for glucose in the regeneration of glycolysis and K influx. In the presence of glucose, adenine plus ribose, and to some extent adenine, but not guanosine, inosine or adenosine triphosphate could substitute for adenosine in the regeneration process. During regeneration there was a marked decrease in the concentration of inorganic phosphate in the cells. Adenosine rapidly disappeared from the medium in which stored duck red cells were suspended both in the presence and in the absence of glucose. The implications of the results with regard to the coupling of K transport with metabolism are discussed.

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POTASSIUM AND SODIUM BINDING BY NUCLEOTIDES

D. C. TOSTESON¹

*Laboratory of Kidney and Electrolyte Metabolism, National Heart Institute,
National Institute of Health, Bethesda, Maryland*

The accumulation of potassium and exclusion of sodium by most living cells has interested physiologists for many years. One plausible explanation of this ionic distribution is that K is transported into the cell in combination with one organic molecule while Na is transported out of the cell in combination with a different reactant. Recent studies suggest that K transport in duck red cells depends on a phosphate containing compound, and under certain circumstances, on a derivative of adenosine (Tosteson and Johnson, '57). Furthermore, there is a considerable body of evidence indicating a specific role for alkali cations in many reactions involving nucleotides (Melchior, '54). The experiments reported in this note were an attempt to find a nucleotide which selectively binds K more than Na or vice versa.

Each of the nucleotides studied was converted to the free acid by passage in the cold through a column of Dowex 50 ion exchange resin which was in the hydrogen form. Two milliliters aliquots of a dilute aqueous solution of each acid were then titrated with NaOH and KOH. For each nucleotide tested, the titration curves obtained with the two different alkalies were identical. The extent of K and Na binding in the resultant alkali nucleotide salt solutions was measured with a cell of the following composition: calomel | sat. KCl agar | NaCl or KCl | cation permeable membrane | Na or K

¹ Present address: Fellow of the National Foundation for Infantile Paralysis, Dept. of Biological Isotope Research, The University of Copenhagen, Copenhagen, Denmark.

nucleotide | sat. KCl agar | calomel. The membranes² were made of collodion impregnated with sulfonated polystyrene by the method of Neihof (Neihof, '54). In every case they yielded a potential difference of 55–56 mV between solutions of 0.1 M and 0.01 M NaCl or KCl. For each nucleotide salt studied, the potential difference developed by the cell was

TABLE 1

| COM- POUND ¹ | PURITY OF COMPOUND | CONCENTRATION OF PURINE OR PYRIMIDINE | CONCENTRATION OF CATION | pH | CATION ACTIVITY COEFFICIENT |
|----------------------------|-----------------------|---|----------------------------|------|-----------------------------------|
| | % | M/l | M/l | | |
| Na ATP | 95 | 0.00246 | 0.0113 | 7.56 | .748 |
| K ATP | 95 | 0.00241 | 0.0114 | 7.55 | .781 |
| Na ITP | 40 | 0.00488 | 0.0161 | 7.80 | .772 |
| K ITP | 40 | 0.00498 | 0.0161 | 7.41 | .775 |
| Na GTP | 40 | 0.00562 | 0.0157 | 7.14 | .760 |
| K GTP | 40 | 0.00572 | 0.0160 | 7.04 | .756 |
| Na UTP | 80 | 0.00284 | 0.0131 | 7.43 | .825 |
| K UTP | 80 | 0.00271 | 0.0131 | 7.40 | .855 |
| Na CTP | 60 | 0.00276 | 0.0125 | 7.62 | .756 |
| K CTP | 60 | 0.00242 | 0.0109 | 7.52 | .795 |
| Na TPN | 95 | 0.00265 | 0.00995 | 7.69 | .904 |
| K TPN | 95 | 0.00260 | 0.0101 | 7.90 | .901 |
| Na CoA | 65 | 0.00265 | 0.0124 | 7.51 | .764 |
| K CoA | 65 | 0.00265 | 0.0124 | 7.47 | .762 |
| Na Isethionate | | 0.00971 | 0.00971 | | .868 |
| K Isethionate | | 0.00942 | 0.00942 | | .950 |

¹ The sources of the compounds were as follows: ATP, Nutritional Biochemical Co.; CTP, CoA, TPN, Pabst Laboratories; UTP, ITP, GTP through the courtesy of W. W. Keilley; isethionate, through the courtesy of J. Moore.

measured in this order under the following conditions: 0.01 M Na or KCl in both chambers of the cell; 0.01 M Na or KCl in left chamber, Na or K nucleotide in right chamber; 0.10 M Na or KCl in left chamber, Na or K nucleotide in right chamber; 0.01 M Na or KCl in left chamber, Na or K nucleotide in right chamber; 0.01 M Na or KCl in both chambers. All measure-

² Kindly supplied to the author by Dr. M. Gottlieb.

ments were made at $25.0 \pm 0.1^\circ\text{C}$. After removal of the nucleotide salt from the cell, the concentration of Na or K present was confirmed by flame photometry. Furthermore, the purity of nucleotides was evaluated by paper electrophoresis at pH 5 in acetate buffer in the cold. The triphosphate spot on the paper was assumed to lie closest to the anode and was further identified by comparison with a simultaneously run sample of relatively pure ATP.³ This spot was eluted from the paper with distilled water and the amount of purine or pyrimidine base present compared with the total amount placed on the paper by measurement of the optical density at the appropriate wave length in the ultraviolet (Kalekar, '47a, '47b).

The activity coefficients of Na and K in the presence of each nucleotide were calculated from the potential measurements and the known activity coefficients of NaCl and KCl (see table). The assumption, implicit in the calculation, that the depression in cation activity by the nucleotide is due to an effect on the activity coefficient rather than actual binding is perhaps justified by the observations of Snell ('55). The relative impurity of the compounds renders the numerical values of the ratios of little use for precise thermodynamical calculations. However, it is clear that none of the nucleotides studied has an appreciable selective affinity for K as compared with Na or vice versa. Similar results were obtained with isethionate, an important anion in squid nerve. The absence of selective alkali cation binding by ATP has been reported previously (Snell, '55; Melchior, '54). It must be concluded that none of these molecules, when free in aqueous solution, act as carriers in the transport of K and Na across cell membranes. The results do not rule out the possibilities that they might participate in alkali cation transport either when bound to a macromolecule in aqueous solution or through a non-aqueous phase.

³ The following abbreviations will be used. Adenosine triphosphate (ATP), inosine triphosphate (ITP), guanosine triphosphate (GTP), uridine triphosphate (UTP), cytidine triphosphate (CTP), triphosphopyridine nucleotide (TPN), co-enzyme A (CoA). The chemical formula of isethionate is $\text{HOCH}_2\text{CH}_2\text{SO}_3\text{H}$.

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OBSERVATION OF MITOCHONDRIA IN LIVING CELLS OF RESPIRATORY MUTANTS OF NEUROSPORA

ELISABETH FREESE-BAUTZ

*Kereckhoff Laboratories of Biology California Institute of Technology,
Pasadena, California*

TEN FIGURES

Earlier cytological observations of cytoplasmic particles in yeast (*Saccharomyces cerevisiae* and *Rhodotorula rubra*) have shown that normally respiring yeast cells (wild type) contain short rod-shaped and/or round mitochondria. In contrast to this, yeasts, with respiratory defects such that they can only ferment, similar to the "petites colonies" described by Ephrussi ('46), have long thread-like mitochondria (Bautz, '55, '56) almost exclusively. The sphere-like yeast cell is not well suited for this type of cytological study; it is easier to observe mitochondria in the long and thin hyphae of other fungi (Bautz, '55b). We therefore decided to look for the presence of such mitochondrial variants in the respiratory mutants of *Neurospora* found by Mitchell and Mitchell ('52, '53). Two types of mutants are considered here: the "poky" strain in which the respiratory deficiency shows cytoplasmic inheritance and the mutant strain "C 117" in which the respiratory deficiency shows Mendelian (nuclear gene) inheritance. Both are spontaneous mutants and result in a slow growth and changes in the cytochrome system (Mitchell et. al. '53, '53b, '54).

At first we wanted to characterize the mitochondria of these respiratory mutants by phase contrast observation, Janus green B and Nadi vital staining, and afterwards we tried to find an explanation of how the change from short to long

mitochondria might come about. The following alternatives seemed most likely: Several short mitochondria are assembled together because their surfaces become sticky, or the mitochondria grow or can still be produced in the respiratory mutants, but their separation is inhibited. In the latter case the production of long threads should occur only in growing hyphae.

We hoped to learn more about this problem by exposing *Neurospora* to acriflavine (euflavine). This substance appears to inhibit the division of cytoplasmic particles; *c. f.* the division of the blepharoblast is inhibited in trypanosomes and flagellates, as shown by Werbitzki ('10) and Robertson ('29); and the petite growth-type in yeast is reported to be produced by an inhibition of the reproduction of cytoplasmic particles (Ephrussi, '53). In addition we hoped to gain further information by the use of varied physiological conditions, *c. f.* oxygen-deficiency, which several investigators had found to change temporarily (modificatory) the shapes of mitochondria in certain plant cells (Meissel, '38, Buvat, '53). For these cells it was assumed that thread-like mitochondria are formed by the cohesion of originally separate, smaller mitochondria.

MATERIALS AND METHODS

Strains of Neurospora crassa:

Wild type P 3178-2a and P 3177-4A *poky* 3627-2a (Mitchell and Mitchell, '52), a respiratory mutant showing cytoplasmic inheritance;

C 117-3672-1A (Mitchell and Mitchell, '53), a respiratory mutant showing Mendelian inheritance.

Culture methods:

Glasswool filtered conidia were usually grown in 10 ml of liquid Fries medium at 25° C. In some experiments, however, Fries medium and a complete medium (Beadle and Tatum, '45) were not only used as liquid media but sometimes they were solidified with 3% agar. The medium of Westergaard

and Mitchell ('47), which favours sexual reproduction, was used for obtaining ascospores. Acriflavine was prepared in phosphate buffer (0.01 mg/ml). It was used in a 1:1 ratio with either minimal medium or buffer. These experiments were done at 25° C. in the dark. To produce oxygen-deficiency preparations were kept under a cover-glass, the edges of which were sealed with wax or vaseline, or, as another means of creating such oxygen-deficiency, argon gas (Linde argon, standard grade) was allowed to bubble through the medium for varying lengths of time.

Staining methods:

Janus green staining, after the method of Sorokin 1938 ('38). One drop of a 1% aqueous solution of Janus green B "Bayer" is added to 50 ml 0.05 M glucose solution. We suspended the mycelia in 5 ml of this solution and examined microscopically in the time between 45 and 90 minutes.

Nadi solution (Perner '52) consists of 2 ml α Naphthol, (0.1% solution in water), 2 ml p-phenyldiamine (0.1% solution in water), and 6 ml of phosphate buffer (5 ml KH_2PO_4 M/15 + 5 ml K_2HPO_4 M/15 + 50 ml water (pH 6.8). The reaction time after addition of this Nadi solution to the cells was always 25 minutes (Marquardt and Bautz, '55).

Equipment:

For the cytological observations a Spencer microscope with normal light and phase contrast was available. The microphotographs were made with a Leica and the corresponding Leitz adapter.

EXPERIMENTAL RESULTS

I. Analysis of the different strains

At the beginning we wanted to be sure that the shape of mitochondria in our Neurospora strains did not depend on the growth media normally used. To check on this we used complete and minimal medium with and without 3% agar and made observations with the phase contrast microscope.

We found that for any one strain, the shape of the mitochondria always remained the same. In addition the variation in media caused insignificant changes in growth behaviour.

Subsequently, we used only liquid minimal medium because it was much easier to take a sample of young hyphae from a liquid culture than from an agar plate.

The following are descriptions of the different strains observed with several cytological techniques:

(1) *Wild type.*

(a) Phase contrast microscopy:

As was expected from our earlier observations (Bautz, '55b), one can see in *Neurospora* three different kinds of particles in the phase contrast microscope. The largest are round or kidney-shaped, show somewhat greater light refraction, and must correspond to the nuclei previously identified (Bautz, '55b) by Feulgen staining and the Carmin squash method. One further sees rod-shaped or round particles which are less light refractive; these are the mitochondria, as we shall show. Finally in large numbers there are seen small plasmagranula which refract the light more than the mitochondria and which we shall call sphaerosomes by analogy to the corresponding particles in higher plants. All three of the above described particles were also seen in the conidiophores, conidia and ascospores. (figs. 1, 2).

(b) Janus green B staining.

A Janus green B staining-reaction is generally accepted as identification of actually respiring mitochondria. In 5 or 12 hours old mycelia, following 45 to 60 minute reaction time, the short rods and the round particles (designated as mitochondria above) were stained a green-blue color. We can be certain of having stained active mitochondria, because after sealing off the coverglass to create oxygen-deficiency the color vanishes. In some, mostly older, vacuole-containing hyphae, all particles and even the cytoplasm itself were stained blue. This typical staining of dying cells was, as expected, not changed by a lack of oxygen.

(c) Nadi-reaction.

With the indophenol blue-reaction for detection of oxydizing enzymes only the small plasmagranula (sphaerosomes) were stained a deep blue. It is known that this staining means a secondary accumulation of indophenol blue, produced by mitochondria, in the lipid-containing granules (sphaerosomes). In some older, vacuolated cells, particles and the cytoplasm were stained blue.

(2) *poky*

(a) In phase contrast.

This mutant with cytoplasmically inherited characters in which the cytochromes and other enzymes are changed, at first grows very slowly but after some days shows increasingly the wild type growth behaviour. We therefore took samples of hyphae after 5 and 12 hours incubation of conidia and also after 1, 2 and 3 days.

The conidia contain, besides nuclei and sphaerosomes, short rod-like structures similar to the mitochondria of wild type and only rarely somewhat longer. But after 5 and especially after 12 hours growth one can see, besides rods, some longer, threadlike particles (fig. 3). Unfortunately, most hyphae already contained many vacuoles after 24 hours and were therefore no longer suitable for cytological purposes. Often the only things recognizable were the cell walls. After the second day the development of conidiophores began, the hyphae showing decreasing amounts of cytoplasm. These conidiophores are again very good for cytological observations, since they do not contain vacuoles. We did not see any threadlike structures in them; only short rods were observed (fig. 4). The same result was obtained when observing ascospore development. In contrast to this intense change of the form of mitochondria, the smaller granula remained unchanged throughout development.

(b) Janus green B staining.

In the conidia and immature ascospores only the lesser light refracting, short, rod-like or round structures could be stained with Janus green B. In the 6 and 12 hours old hyphae

some of the short rods and also some of the shorter threads were stained blue-green. They lost their colour again under oxygen-deficiency. In the older hyphae usually everything was stained as in the wild type and remained so under oxygen-deficiency. The conidiophores, young asci and young ascospores showed staining of short rod-like and round mitochondria.

(c) Nadi-reaction.

In young *poky* hyphae the Nadi-reaction stained the plasma-granula (or sphaerosomes) exclusively, mostly with light blue colour. In older hyphae everything was stained.

(3) *C 117 Strain*

(a) Phase contrast.

In the very slowly germinating conidia one can clearly see elongated structures (fig. 5). After 12 to 24 hours growth we saw in many hyphae tremendously long thin threads, which in some extreme cases reached $3/4$ of the cell length (fig. 6). The length of the threads varied greatly from cell to cell, and within each cell. In some cells we could also see some short rod-like mitochondria. The threads were also found in the conidiophores, but they were generally shorter than in the hyphae. (figs. 7, 8).

The threads extended mostly longitudinally in the cells, presumably in the direction of the streaming cytoplasm. In some cases we could even see a movement of the threads. Figures 7, 8 show two different stages in the movement of a single thread.

(b) Janus green staining.

True Janus green vital staining, which disappears under oxygen-deficiency, could only be observed for the small numbers of rods and short threads. We never observed staining of the extremely long threads.

(c) Nadi-reaction.

Only the plasmagranula were stained an indophenole light blue. The colouring in older hyphae was more pronounced.

II. Application of acriflavine

(1) Growing Hyphae (dividing cells).

In order to observe the action of acriflavine upon growing cells we inoculated the conidia into a 1:1 mixture of acriflavine and minimal medium. The mycelium grew slowly.

(a) Wild type.

After 12 hours of growth we observed in the phase contrast microscope that some of the hyphae contained extremely long threads instead of the short rod-shaped mitochondria (figs. 9, 10). The long threads looked similar to those normally occurring in the mutant C 117. The longer threads did not stain with Janus green, although some of the shorter threads became somewhat stained. The Nadi-reaction gave only pale blue stained granula.

(b) *poky*.

In the small number of still growing hyphae we found, in addition to typical range of variation in mitochondrial size, now some very long threads. Janus green and Nadi-reaction gave the same results as found with wild type.

(2) Resting Hyphae (non-dividing cells).

We left the conidia for 12 hours in minimal medium and then transferred the grown mycelium into an acriflavine-buffer solution. One could see clearly, both macroscopically and in the microscope, that the cells no longer grow in the buffer.

(a) Wild type.

After 5 hours in the acriflavine-buffer the cells appeared unchanged. We saw typical wild type hyphae with short rod-like or round mitochondria. After 12 hours the mitochondria were still unchanged. However, the cytoplasm became granular or flocculent, especially after 24 hours.

(b) *poky*.

The same results were obtained as with wild type. The mitochondria retained the shapes found in typical *poky*.

(3) Removal from acriflavine.

We next attempted to discover whether the effect of acriflavine upon the cells was permanent or transient. At

intervals, two samples were taken from mycelia growing in minimal medium plus acriflavine. One of each pair of the samples was placed in minimal medium and the other in buffer.

After 12 to 24 hours in minimal medium all the young hyphae, especially the growing tips, had the typical wild type mitochondria. Only in some of the old cells could we still detect some threadlike mitochondria. The samples placed in buffer were first washed several times with buffer. Under these conditions little or no growth occurred. In these non-growing hyphae the long threadlike mitochondria remain unchanged.

III. Effect of oxygen-deficiency

In order to see if the shape of mitochondria in *Neurospora* changes under oxygen-deficiency, as has been described for some other plants (Buvat, '53, Meissel, '38), we have observed wild type and *poky* under a sealed coverglass and also after treatment with argon gas.

(a) Wild type.

A small sample of 6 hour old mycelium was put under a coverglass, the edges of the glass tightly sealed, and the preparation kept in a moist chamber. After 3, 5, and 12 hours no change of the mitochondria could be seen with phase contrast. The same result was obtained after bubbling argon for 12 hours through a suspension.

(b) *poky*.

No change of mitochondria was observed under sealed coverglass and in argon.

(c) Removal of oxygen-deficiency.

In all cases of oxygen-deficiency the growth was almost completely stopped after a short time. This was expected because *Neurospora* is strictly aerobic. However, after 12 hours oxygen-deficiency the cells are evidently not dead. This was shown by the fact that the hyphae of both the *poky* and wild strain grew rapidly and produced conidia, when they were once again exposed to air.

DISCUSSION

As with our earlier observations in yeast (Bautz, '55) we have now shown for *Neurospora* that the shape and the cytochemical reactions of mitochondria, the sites of respiratory enzymes, are changed in the respiratory mutants. While normally respiring cultures contain short rod-like or round mitochondria one can observe longer rods or extremely long threads in the mutant strains. In order to identify these threads as active mitochondria we stained with Janus green. The definitive Janus green mitochondrial staining disappears in oxygen-deficiency. This latter reaction indicates the sites of active oxygen metabolism (Sorokin, '38, Drawert, '53, Bautz, '56). We found that the wild type mitochondria show these reactions. On the other hand in the mutants only some of the short threads (often present in very small numbers) showed any staining. The long threads in the mutants were never stained by Janus green B. We found that in cells containing primarily long thread-like mitochondria, secondary staining of plasmagranules (sphaerosomes) with indophenol blue resulting from the Nadi-reaction for oxydizing enzymes was much less intense than in cells containing short mitochondria. This provides further evidence of depressed oxygen metabolism in the mutants.

These results are in agreement with physiological and biochemical observations made by Mitchell and his collaborators ('53, '53b, '54) which showed that the mutant strains have much less respiratory activity than wild type and lack some enzymes important for respiration. While the mutant C 117, with Mendelian inherited characters, always keeps this very pronounced deficiency, the *poky* strain, which shows cytoplasmic inheritance, changes its appearance after several days growth increasingly approaching that of the wild type. In our cytological observations we saw that after 2 — 3 days the newly produced conidiophores in *poky* contain short mitochondria exclusively. Comparing the biochemical and physiological results of Mitchell et al. ('52, '53) and our cyto-

logical observations we can state, that in the respiratory deficient mutants of *Neurospora*, the defects are associated with the presence of thread-like mitochondria.

It could be possible that the thread-like structures are not mitochondria but are other cytoplasmic particles and the respiratory deficiency would be due to the fact that there are not enough of the short rod-like mitochondria. But because light refraction and the width of the short and the long threads are always the same, it seems more probable that the long threads are produced from mitochondria which have lost their typical properties. This raises the question of how these alternatives can be distinguished. We thought to get an answer by trying to produce such filaments and by looking to see how they are formed, for it is already known that one can produce filamentous mitochondria by experimental means.

For example, Meissel has shown in yeast ('38) and Buvat ('53) (Sorokin, '55) in epidermal cells of higher plants, that oxygen-deficiency causes the appearance of long thread-like mitochondria. Buvat and Sorokin have proposed that they are created by the linear aggregation of short mitochondria.

Using the same experimental technique as Buvat and Sorokin (sealing the coverglass) and also producing oxygen-deficiency by replacing air with argon gas, we never observed any change of the mitochondrial shape in *Neurospora*. On the other hand we succeeded in getting a marked change in mitochondrial shape in the cells of some wild type and *poky* hyphae, when they were grown in minimal medium plus acriflavine which is known c.f. to inhibit the division or reproduction of cytoplasmic particles (Werbitzki, '10; Robertson, '29; Ephrussi, '46). In this way we obtained extremely long, thread-like mitochondria, similar to those found normally in the C 117 mutant. This change was not permanent, and active growth was required for a change in any direction. This was shown by the fact that there was no change from the normal shape of mitochondria in wild type mycelia placed in acriflavine plus buffer (no growth). The reverse change from threadlike to normal wild type was prevented when

mycelia were removed from acriflavine plus medium and placed in buffer.

These results show that the production of threads under the influence of acriflavine occurs only during the increase of cytoplasm and during cell growth. It therefore seems less probable in our case that several short mitochondria assemble to form a long thread, but more probable that they can grow or duplicate in growing cells and the acriflavine prevents their separation (or mitochondria originating from ultraviolet "promitochondria" keep on growing longer than normally).

It is an important fact that the threads produced under the influence of acriflavine do not exhibit the same staining reactions as normal wild type mitochondria, but they do appear similar to the threads observed in the mutant strains.

These experiments on the production of filaments under the influence of acriflavine provide further support for the hypothesis that the filaments are really changed mitochondria. But by what processes are the thread-like mitochondria produced in the mutant strains?

It is of great importance that a cytoplasmic mutation can produce the same or similar properties as a gene mutation. The cytoplasmic mutant *poky* is more variable with respect to the morphology and the cytochemical reactions of the mitochondria and more variable in growth and respiratory characteristics (Haskins et al. '53) in spite of the fact that its pattern of change is highly reproducible. But these differences between the cytoplasmic and the gene mutant are not surprising, for the greater variations are typical for all properties which have been shown to be directed by the cytoplasm (Oehlkers, '52, '56, etc.). Since nuclear genes may act quite indirectly, the mitochondria might not be the first link in a reaction chain but a later one, a cytoplasmic mutation may easily affect the same system. It is possible that in the mutants the nuclear genes as well as the cytoplasmic units produce substances that have a thread-producing action sim-

ilar to that of acriflavine. It remains to be established whether the same substances that cause the morphological changes also give rise to the observed differences in the cytochrome systems of the *Neurospora* mutants.

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SUMMARY

We have shown that the shape and cytochemical reaction of mitochondria are changed in respiratory mutants of *Neurospora* in the cytoplasmic mutant *poky* as well as in the gene mutant C 117. By observations with the phase contrast microscope, we saw that the normal respiring wild type contains round or short rod-shaped mitochondria, the cytoplasmic mutant *poky*, rod-shaped and short threads and the nuclear-gene mutant C 117, very long thread-like mitochondria. With Janus green B staining reaction only the round rod-shaped and short threads could be stained and therefore only these can be actually respiring mitochondria. With the Nadi-reaction wild type showed a deep blue staining, the mutants only light blue. This indicates that the mutants have a lower rate of oxygen consumption.

With acriflavine we were able to produce long thread-like mitochondria in some wild type and *poky* hyphae, but only when the cells were growing in medium containing the acriflavine.

Under conditions of oxygen deficiency we did not obtain a change of the mitochondrial shape of wild type *Neurospora*.

In the discussion we have attempted to explain, how the thread-like mitochondria might be produced in *Neurospora* strains.

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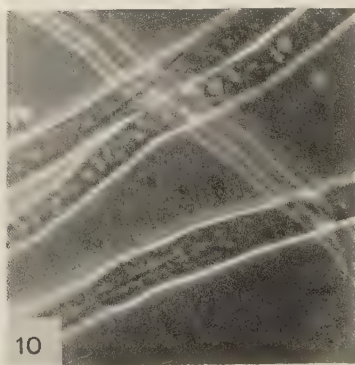
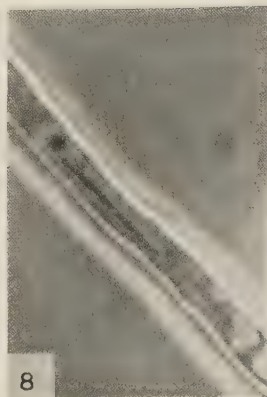
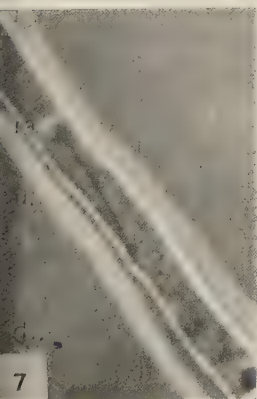
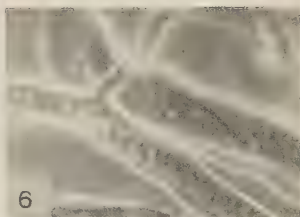
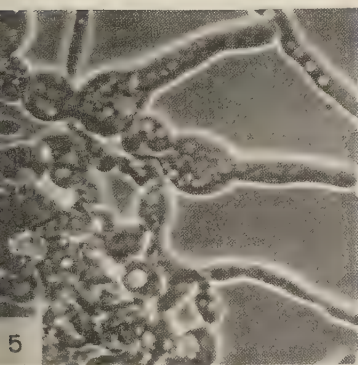
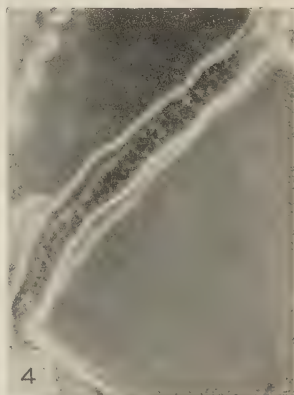
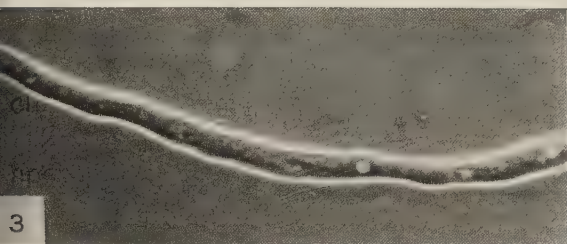
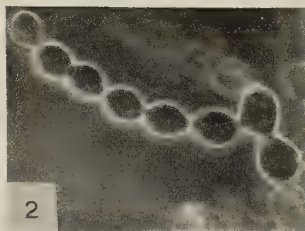
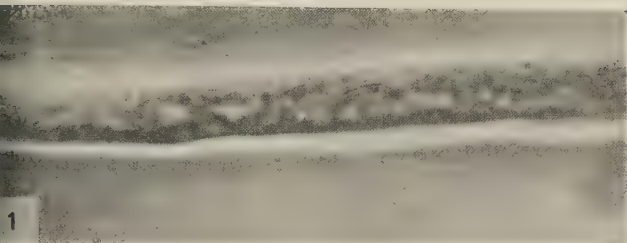
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PLATE 1

EXPLANATION OF FIGURES

- 1 Wild type *Neurospora* with only short rod-shaped and round mitochondria.
- 2 Wild type-conidia with short and round mitochondria.
- 3 *Poky* hyphae shows three more thread-like mitochondria besides the short rod-like forms.
- 4 *Poky* conidiophore shows only short and round mitochondria.
- 5 C 117, germinating conidia, with more or less thread-like mitochondria.
- 6 C 117 — hyphae, one of them shows a long thread.
- 7 C 117 — cell, with a long thread-like mitochondrion.
- 8 The same cell as in fig. 7 after a movement of the thread-like mitochondrion.
- 9 Wild type after 12 hours growth in acriflavine plus medium showing thread-like mitochondria similar to those of C 117 (Figs. 6, 7, 8).
- 10 Threads in wild type produced by acriflavine.



OBSERVATIONS ON THE POLYSACCHARIDES OF AQUATIC SNAILS¹

PATRICIA McMAHON, THEODOR VON BRAND, AND M. O. NOLAN

*U. S. Department of Health, Education, and Welfare Public Health Service
National Institutes of Health, National Institute of Allergy and
Infectious Diseases,² Bethesda 14, Maryland*

THREE FIGURES

In most studies of endogenous carbohydrate metabolism of invertebrates the only polysaccharide considered is glycogen. This polysaccharide is the most widely distributed, and in many animals it is the only polysaccharide occurring in appreciable amounts. However, in snails such studies are complicated by the occurrence of another polysaccharide, galactogen, in addition to glycogen. Galactogen has been found in the albumen gland and eggs of *Helix* (May, '31, '34 a, b; Baldwin and Bell, '38), *Lymnaea* (Holm, '46), *Viviparus* and *Ariophanta*, as well as in the uterus and eggs of *Pila* (Meenakshi, '54). Adequate proof has been presented that the galactogen of *Helix* consists of d- and l-galactose units (Bell and Baldwin, '40, '41; May and Weinland, '54) but the question whether the polysaccharide consists of highly branched chains or not, is controversial (O'Colla, '53). The assumption has been made that the galactose polysaccharide occurring in aquatic snails is identical with that occurring in the terrestrial snail *Helix*, but the criteria used (demonstration of galactose, negative iodine test) are insufficient to prove this assumption.

¹ Part of the work reported herein was submitted by P. McMahon to the Graduate School of Georgetown University in partial fulfillment of the requirements for the degree of Master of Science.

² Laboratory of Tropical Diseases.

This paper presents a more detailed study of the sugars occurring in the polysaccharides of aquatic snails, with particular emphasis on those of the reproductive tract and eggs.

MATERIAL AND METHODS

Snails

Whole snails and isolated organs were employed as follows:

1. *Australorbis glabratus*, Venezuelan strain, laboratory-reared. Whole adult snails, isolated albumen glands, and egg clutches of various ages were used.

2. *Lymnaea stagnalis*, laboratory-reared from Wisconsin specimens. Whole adult snails, freshly hatched snails, isolated albumen glands, muciparous glands, oothecal glands, seminal vesicles, upper and lower prostates, entire egg clutches of various ages, eggs, membranes, and mucus isolated from fresh clutches were employed.

3. *Aplexa nitens*, laboratory-reared from Texan material. Only isolated albumen glands were used.

4. *Lanistes boltenianus*, laboratory-reared from Egyptian specimens. This species, in contrast to the three former, is dioecious. Male and female specimens were therefore analyzed separately. The isolated uterus, whole egg clutches, and isolated eggs were also studied.

5. *Pomacea zeteki*, laboratory-reared. The original specimens were obtained from a local pet shop; the actual place of origin was probably Panama. Of this dioecious species, entire male and female adults were analyzed, as well as freshly hatched snails, the isolated uterus of adult females, and egg clutches of various ages.

6. *Otala lactea*, imported Moroccan specimens. Only isolated albumen glands were studied.

7. *Helix aspersa*, Michigan specimens. Entire snails were used. The last two species were terrestrial, all others aquatic.

GENERAL METHODS

Sexing of the dioecious species was difficult, because no distinctive external sex character was found. Snails were

thoroughly chilled on ice, rapidly opened, and sex determined by inspection of the internal organs. The entire snail was then immersed in a 60% solution of KOH. Similarly, for the isolation of internal organs, chilled snails were dissected on ice under a dissecting microscope and isolated organs transferred to the alkaline solution.

In order to collect egg clutches of known age, the aquarium was cleared of all eggs and those laid in the following 24-hour period were outlined on the outside of the tank with a grease pencil. They were used either immediately or after specified periods of development. A large number of freshly laid egg clutches of *Lymnaea* were divided into three components for separate analysis. The outer membrane was isolated by cutting the clutch and scraping the mucus and eggs from the membrane. The mucus-embedded eggs were placed in dilute anti-formin solution and the dissolved mucus removed from the eggs by means of a pipette. The larger eggs of *Lanistes* were freed of mucus in essentially the same way. The eggs of this species are embedded in viscous mucus, but are not surrounded by a distinctive membrane.

In the case of *Lymnaea* and *Pomacea*, freshly hatched snails were also used. The former species lays its egg clutches below the water line. Clutches were removed from the aquarium and placed in petri dishes containing clean water. *Pomacea* deposits its egg clutches above the water line and frequently on the plate covering the aquarium. In the latter case, the plate was removed and placed over a battery jar containing fresh water into which the young snails dropped upon hatching. The young *Lymnaea* and *Pomacea* were collected once or twice a day as they hatched; no extraneous food material was available from the time of hatching until collection.

Polysaccharides were isolated essentially according to Pflueger's procedure. Starting material was heated with 60% KOH solution for one to two hours in boiling water and polysaccharides precipitated with ethanol. Purification was achieved by redissolving the material in water, acidifying with 4% aqueous HCl, removing impurities by centrifugation,

and reprecipitating with ethanol. After two or three further precipitations the polysaccharides were usually pure white. They were washed with absolute ethanol and anhydrous ether and dried in a vacuum desiccator over CaCl_2 . This purification procedure, with minor modifications as to the number of reprecipitations necessary, was adequate for most polysaccharides. When whole *Australorbis* was used, however, the black pigment present in this species interfered since it was so finely dispersed that it could not be removed completely by centrifugation. In this case the aqueous polysaccharide solution was filtered through a Berkefeld filter which effectively retained the pigment. Purified polysaccharides were subjected to analysis by paper chromatography and paper electrophoresis as detailed in the following sections.

Quantitative determinations were made of the total polysaccharides of some isolated organs and egg clutches of various ages. For this purpose, material isolated essentially as described above was hydrolyzed at 100° for two to three hours in a 2.5% solution of HCl and the reducing sugars were determined according to the method of Hagedorn and Jensen ('23). When little material was available, von Brand's ('36) micro-modification of Pflueger's method was used. The material was calculated as galactose polysaccharide since, as will be shown below, galactose was the predominant component. The reducing value of galactose was taken from Weise and von Brand's ('33) table. In the case of egg clutches, dry weights were determined by drying the clutches at 110° to constant weight.

Paper chromatography

Samples were prepared for chromatographic analysis by hydrolyzing 10 mg of polysaccharide with 1.0 ml of 1 N H_2SO_4 for 3 hours at 100° and neutralizing with solid barium carbonate. Removal of the barium sulfate precipitate by centrifugation yielded a clear, neutral, relatively salt-free supernatant solution. Ten μl of this solution, corresponding to 0.1 mg of polysaccharide, was spotted on the chromatographic paper.

Triple ascending development was employed with the use of Whatman no. 1 chromatographic paper stapled to form a cylinder. Museum jars served as chromatographic chambers with the stationary phase of the developing solvent contained in a beaker centered on the floor of the jar and 100 ml of the mobile phase poured directly into the jar around the beaker.

Three solvent systems were used: butanol, pyridine, water, benzene (5:3:3:1) (Albion and Gross, '50); butanol, ethanol, water (4:1:5) (Partridge, '46); and butanol, acetic acid, water (4:1:5) (Partridge, '48). The location of the sugars on the chromatograms was established by the use of aniline phthalate (Partridge, '49) which was found to be sensitive to 2 μ g of glucose or galactose. The less sensitive phloroglucinol spray of Horrocks and Manning ('49) was used to indicate the nature of the sugars. The presence of amino sugars was tested by use of an alcoholic ninhydrin spray on chromatograms developed in the acidic solvent and by the modified Elson and Morgan reagent (Partridge, '48) on chromatograms developed in the neutral or basic solvents. A spray of resorcinol and hydrochloric acid in ethanol was used to detect the presence of ketoses. A component of the polysaccharide hydrolyzate was considered identified when its migration in the three solvents and its reactions with all spray reagents were identical with those of a known sugar.

If the supply of polysaccharide permitted, estimation of the relative amounts of the components was made by elution of the sugars from chromatograms. Approximately 0.2 ml of hydrolyzate was applied along the starting line of the chromatogram. After development, a half-inch strip, the length of the cylinder, was cut from the center and from each side of the chromatogram and sprayed with the aniline phthalate reagent. The areas of the reducing sugars were noted and corresponding areas on the unsprayed parts of the chromatogram were cut from the paper, placed in beakers, and washed with 3.0 ml distilled water. After filtering through Whatman no. 50 filter paper to remove fibers of chromatographic paper, the washings were diluted to 10.0 ml. This solution, or

portions of it, was analyzed for reducing sugars according to the method of Hagedorn and Jensen ('23). Solvent-paper blanks were prepared and their reducing values subtracted from those of the eluted sugars. The amounts of sugar present were calculated from the table given by Weise and von Brand ('33), or, in the case of fucose and glucosamine, from standard curves. Recovery of galactose standard by this technique was 97%.

Paper electrophoresis

The Spinco paper electrophoresis unit with Durrum-type cell was employed with Whatman 3 MM paper strips and 0.1 M borate buffer, pH 9.8. Ten μ l of an aqueous solution containing 1.0 mg polysaccharide was striped on the paper and subjected to 210 V., 4 M.A. for four to six hours at room temperature. On removal from the cell, the paper strips were submerged in 95% ethanol to fix the polysaccharides, and stained by the periodate-Schiff method of K  iw and Gr  nwall ('52). The electrophoretic migrations of the polysaccharides were compared with those of commercial glycogen (Nutritional Biochemicals Corporation) which migrated 1/4 inch toward the anode in 4 hours, and galactogen isolated from the albumen gland of *Otala lactea* which migrated 2 inches.³

When sufficient polysaccharide was available, the components of each of the electrophoretic zones were determined by elution in a manner similar to that described for elution of the chromatographic papers. The eluates of eight papers were pooled, the polysaccharides precipitated with ethanol, hydrolyzed, and chromatographed as described above.

³ There is a distinct difference between our methods and those of Geldmacher-Mallinckrodt and Weinland ('53). These authors separated glycogen and galactogen on silk by electrophoresis and find that both polysaccharides migrated toward the cathode, the migration of galactogen being slower than that of glycogen. In our experiment we find the opposite: polysaccharides migrate toward the anode and galactogen migrates faster than glycogen. This is due to the different buffers employed; the above authors used veronal buffer while we used borate, which forms a borate-polysaccharide complex.

RESULTS

Biological data

Since no relevant data on the egg laying habits of *Lanistes boltenianus* and *Pomacea zeteki* are available in the literature, they are recorded here.

The snails of both species were maintained at 26° to 27° in battery jars containing standing tap water⁴ in which algae grew, and small amounts of fine sand, and calcium carbonate. The snails were fed lettuce leaves (fresh leaves, or leaves scalded with boiling water), fish food, and occasionally a mixture of equal parts of fish food and yeast. Gentle aeration was provided at all times and the jar contents were changed weekly.

Under these conditions, *L. boltenianus* began to lay eggs at 6 to 7 months of age. The egg clutches were deposited below the water line, usually on the glass of the jar. The egg clutches were elongated and the eggs were lined up usually in two rows along the clutch and embedded in viscous mucus. In one instance, the oviposition was observed from start to finish, lasting 3 hours and 15 minutes. This particular clutch contained 109 eggs. A wide variation in the size of the clutches and number of eggs per clutch occurred. The average number of eggs per clutch (125 clutches) was 60 with extremes of 12 and 135. The time elapsing between oviposition and hatching was 13 to 15 days, all young snails of a given clutch hatching within two days. Eggs which were exposed to fluctuating temperatures (warm during day, cold at night) were delayed in their development, requiring up to 25 days for hatching, which then continued for 10 days.

Pomacea zeteki also began to lay eggs when 6 to 7 months old. The big (e.g., one large clutch measuring 55 × 12 × 10 mm) and compact clutches were laid above the water line. They were opaque, and a counting of the eggs was impossible since they occurred in several layers. Minimal numbers for two clutches (derived by counting the snails hatched) were

⁴ Tap water was allowed to stand in large cylinders for more than 24 hours to facilitate the escape of chlorine.

164 and 253. The snails started to hatch 11 to 13 days after oviposition and continued to hatch for 2 to 3 days. The young snails crawled around on the clutch and apparently fed on the viscous mucus-like material which cemented the eggs together. After variable periods the snails dropped into the water.

Since the egg laying habits of the other species are well known, they are not detailed here. The eggs of *Australorbis glabratus* hatched after about 7 days development, while those of *Lymnaea stagnalis* required 10 to 11 days.

Chromatographic and electrophoretic analysis

Australorbis glabratus: Galactose and fucose were the only sugars detected on chromatograms of hydrolyzed polysaccharide from 1- and 3-day-old egg clutches. The hydrolysate of polysaccharide from 6-day-old clutches, in which the snails

TABLE 1

*Approximate composition of some polysaccharides
from aquatic snails¹*

| SOURCE | COMPONENT SUGARS | | | | |
|-------------------------------|------------------|-----------|---------|------------------------------------|--------|
| | Glucosamine | Galactose | Glucose | Unidentified sugar ² | Fucose |
| <i>Australorbis glabratus</i> | | | | | |
| egg clutches | | | | | |
| 1 and 3 days old | | 96% | | | 4% |
| 6 days old | | 88% | 8% | | 4% |
| <i>Lymnaea stagnalis</i> | | | | | |
| egg clutches | | | | | |
| 1 and 3 days old | 5% | 77% | | 5% | 13% |
| 6 days old | 2% | 84% | < 1% | 5% | 8% |
| 9 days old | 5% | 81% | 2% | 3% | 9% |
| freshly hatched snails | | 70% | 30% | | |
| <i>Lanistes boltenianus</i> | | | | | |
| isolated eggs | | 90% | | | 10% |
| <i>Pomacea zeteki</i> | | | | | |
| egg clutches | | | | | |
| 1 and 6 days old | | 95% | | | 5% |

¹ Expressed as percent of total sugars obtained by elution of paper chromatograms.

² Reducing power arbitrarily assumed to be similar to that of fucose.

were almost ready to hatch, contained glucose in addition to galactose and fucose. (table 1.)

The whole adult snails yielded polysaccharide qualitatively similar to that of 6-day-old egg clutches, but the glucose spot on the chromatograms was by far heavier than those of the other sugars. (fig. 1). Polysaccharide obtained from isolated

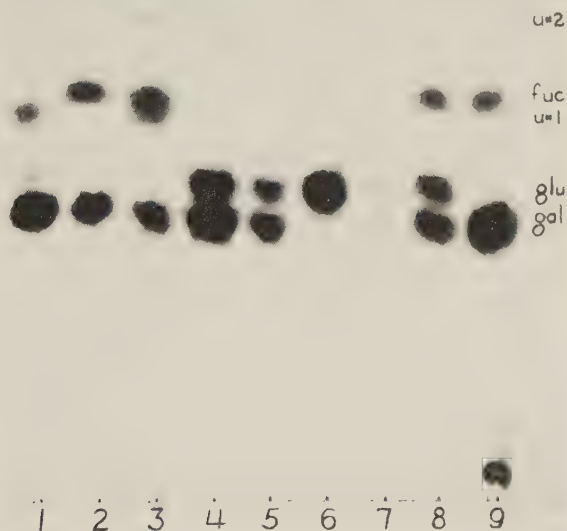


Fig. 1 Paper chromatogram of hydrolyzed polysaccharide from snails: triple ascending development in butanol, pyridine, water, benzene (5:3:3:1), sprayed with aniline phthalate reagent. (1) *Lymnaea stagnalis* mucus, (2) galactose, fucose standard, (3) *Lymnaea stagnalis* membrane, (4) *Helix aspersa*, whole adult snail, (5) galactose, glucose standard, (6) *Australorbis glabratus*, whole adult snail, (7) *Pomacea zeteki*, freshly hatched snails, (8) galactose, glucose, fucose standard, (9) *Lanistes boltienianus* isolated eggs. Legend: gal., galactose; glu., glucose; u no.1, unidentified hexose-like sugar; fuc., fucose; u no. 2, unidentified reducing substance. Although in the photograph the spots from fucose and the unidentified hexose-like sugar cannot be distinguished from one another when they occur together (sample 3), the fucose spot is brown while the unidentified sugar spot is red-brown. High salt concentration in sample no. 9 caused salt spots near the origin and between the galactose and fucose spots. Amino sugars react with aniline phthalate to produce spots too light for photographic reproduction.

albumen gland contained only glucose and galactose, the latter sugar being the predominant one. Electrophoretic migration of albumen gland polysaccharide indicated that it was a combination of glycogen and a somewhat larger amount of galactogen.

TABLE 2

*Separation of sugars by triple ascending development in acidic, basic, and neutral solvents on Whatman No. 1 paper.
The figures designate inches traveled by the sugars*

| SUGARS | SOLVENTS ¹ | | |
|--------------------------|---------------------------------------|-----------------------------------|---|
| | Butanol 4 Acetic acid 1 Water 5 | Butanol 4 Ethanol 1 Water 5 | Butanol 5 Pyridine 3 Water 3 Benzene 1 |
| Galactosamine | 2.4, 3.5 | 1.4 | 3.0, 3.5 |
| Glucosamine ² | 2.5, 3.3 | 2.5, 3.1 | 3.5, 4.0 |
| Galactose | 3.5 | 2.9 | 4.4 |
| Allose | 3.5 | 3.5 | 5.3 |
| Glucose | 3.7 | 3.1 | 5.4 |
| Gulose ³ | 3.9 | 3.2 | 5.6 |
| Mannose | 4.0 | 3.7 | 5.7 |
| D-glycero-D-glucose | 4.1 | 2.6 | 4.5 |
| Altrose | 4.4 | 4.3 | 6.3 |
| Idose | 5.0 | 5.0 | 7.6 |
| Talose | 5.1 | 4.7 | 7.2 |
| Unkown ⁴ | 5.3 | 5.0 | 6.8 |
| Fucose | 5.9 | 5.0 | 7.4 |

¹ Solvent components are expressed as volumes.

² Glucosamine HCl.

³ Gulose CaCl₂.

⁴ Unkown sugar from mucus, membranes, muciparous and oothecal glands of *Lymnaea*.

Lymnaea stagnalis: On hydrolysis, polysaccharide isolated from all egg clutches yielded glucosamine, galactose, fucose, and an unidentified sugar which migrated with fucose on chromatograms developed with the neutral solvent, but migrated just behind fucose on chromatograms developed with the acidic or basic solvents. This sugar gave typical aldohexose reactions with all spray reagents but could not be identified as

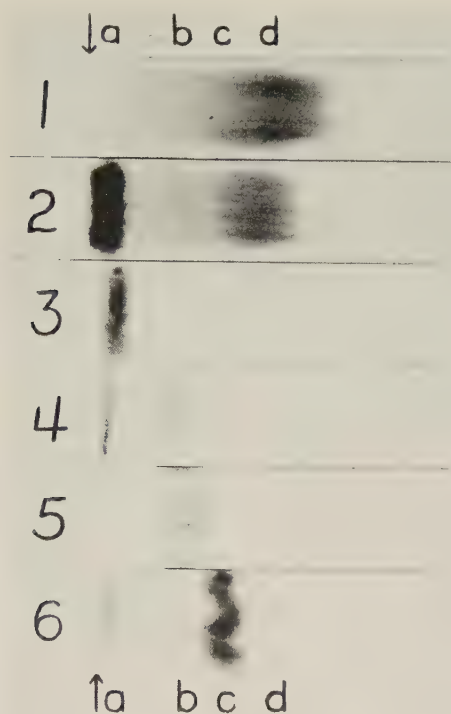


Fig. 2 Electrophoretic migrations of polysaccharides from aquatic snails compared with those of glycogen (a), and galactogen of terrestrial snails (d); borate buffer 0.1 M, 210 V, 5 m. a., 6 hrs. All polysaccharides migrated toward the anode, arrow indicates origin. Sources: (1) *Octala lactea* albumen gland; (2) *Helix aspera*, whole snail; (3) glycogen; (4) *Pomacea zeteki*, freshly hatched snails; (5) *Lymnaea stagnalis* mucus; (6) *Lanistes boltienianus* isolated eggs. Letters are to facilitate comparison of the relative rates of migration.

any aldohexose by its migrations on chromatograms (table 2).⁵ In addition to the sugars mentioned above, glucose appeared in the hydrolyzates of polysaccharides isolated from 6 and 9-day-old egg clutches, the latter being nearly ready to hatch (table 1).

⁵ Dr. Gilbert Ashwell, National Institute of Arthritis and Metabolic Diseases, conducted a series of specific color reactions on a concentrated eluate of this sugar. The cysteine-sulfuric acid tests of Dische indicated a hexose-like character but failed to identify the sugar as a specific hexose. The unidentified sugar did not react as any of the commonly occurring ketohexoses or ketopentoses, nor did it appear to be a pentose when subjected to the orcinol or cystine-carbazole reactions.

Polysaccharides obtained from isolated mucus of egg clutches migrated 1.3 inches from the origin when subjected to paper electrophoresis for 4 to 6 hours (fig. 2) and on hydrolysis yielded galactose, glucose, glucosamine, and the unidentified hexose-like sugar (fig. 1). Egg clutch membrane contained polysaccharide which migrated one inch from the origin during paper electrophoresis. It contained galactose, fucose, glucosamine, the unidentified hexose-like sugar and a trace of a second unidentified reducing substance whose migration was similar to that of a uronic acid lactone (fig. 1). However, this reducing substance did not give any of the typical lactone reactions with spray reagents and the small amount present precluded further identification.⁶ It should be emphasized that the unknown sugars are not artifacts due to treatment with alkali during isolation of the polysaccharide. This was shown by hydrolyzing membranes directly with acid, without any treatment with alkali. Chromatograms of this hydrolyzate showed exactly the same constituent reducing substances as those prepared from alkali-treated material. The former contained, of course, many ninhydrin positive spots in addition to the sugars.

The muciparous gland yielded polysaccharide containing the same sugars as that from the mucus. Electrophoresis revealed, however, that the former contained glycogen as well as a polysaccharide with the same electrophoretic migration as the mucus polysaccharide. The oothecal gland contained glycogen and a polysaccharide with the same electrophoretic migration as that found in membrane. Chromatography of the hydrolyzate of the polysaccharide mixture demonstrated the presence of glucose in addition to the sugars obtained from membrane polysaccharide. Albumen gland polysaccharide, on the other hand, yielded only galactose and was electrophoretically homogeneous.

⁶ To test whether this reducing substance was the fucoketose reported by Wilkinson Dudman and Aspinall ('55) to be formed by prolonged contact of fucose with BaCO_3 , fucose was dissolved in 1 N H_2SO_4 , the solution neutralized with an excess of BaCO_3 , and allowed to stand at room temperature 48 hours. Chromatography of the supernatant solution did not reveal any spot corresponding to that of the unidentified reducing substance.

Polysaccharide from the upper prostate contained galactose, glucose, and fucose, while that of the lower prostate had the same sugars as that found in the mucus and muciparous gland. The polysaccharide isolated from the seminal vesicle had the same constituents as that isolated from the oothecal gland.

Galactose and glucose were evident on chromatograms of the hydrolyzed polysaccharide of freshly hatched snails, but fucose could not be detected. Fucose did appear, however, on chromatograms of polysaccharide hydrolyzates of adult snails. The unidentified sugars and glucosamine, which theoretically should be present in the polysaccharide of whole snails, also failed to appear on chromatograms. Apparently their concentrations were too low.

Lanistes boltenianus. Polysaccharide obtained from isolated eggs and from fresh whole egg clutches was electrophoretically homogeneous, migrating 1.5 inches in 4 hours (fig. 2). Galactose and fucose were obtained on hydrolysis (table 1). Ten-day-old egg clutches contained glycogen as well as a galactose-fucose polysaccharide. Whole mature females and isolated uteri also yielded these two polysaccharides. Whole mature males, on the other hand, contained only glycogen.

Pomacea zeteki. Egg clutches one and six days old contained polysaccharide which on hydrolysis yielded galactose and fucose (table 1). This polysaccharide was electrophoretically homogeneous and could be distinguished from that occurring in *Lanistes boltenianus* by slower electrophoretic migration, 1.25 inches in 4 hours (fig. 2). This polysaccharide was accompanied by glycogen in 10-day-old egg clutches, freshly hatched snails, mature females, and isolated uteri. Only glycogen was found in mature males.

Otala lactea. Isolated albumen gland contained only galactogen which upon electrophoresis migrated 2 inches in 4 hours (fig. 2).

Helix aspersa. The polysaccharides of whole snails separated upon electrophoresis into two components: glycogen and galactogen (fig. 2), the latter migrating at the same rate as the galactogen isolated from the albumen gland of *Otala lactea*.

Quantitative observations

Table 3 summarizes quantitative polysaccharide determinations made of structures of the reproductive glands that could be isolated in sufficient quantity to give reliable values. It is evident that the albumen gland always contains a high percentage of polysaccharide. This is in agreement with data available in the literature concerning *Helix* (May, '34) and

TABLE 3

Polysaccharides in components of the female reproductive system of aquatic snails. The polysaccharides are expressed in percent of the fresh tissues, the weight of the individual structures in mg. The figures behind the \pm signs are the standard error of the mean.

| STRUCTURE | | AUSTRALORBIS GLABRATUS ¹ | APLEXA NITENS ² | LYMNAEA STAGNALIS ³ | LANISTES BOLTEN- IANUS ⁴ |
|------------------|-----------------|--|-------------------------------|-----------------------------------|---|
| Albumen gland | Polysaccharides | 12.8 \pm 0.9 | 6.0 \pm 1.0 | 15.1 \pm 1.5 | |
| | Weight | 19.4 \pm 3.0 | 7.2 \pm 0.5 | 65.5 \pm 3.5 | |
| Oothecal gland | Polysaccharides | | | 10.6 \pm 2.1 | |
| | Weight | | | 48.2 \pm 3.8 | |
| Muciparous gland | Polysaccharides | | | 1.5 \pm 0.1 | |
| | Weight | | | 17.6 \pm 1.1 | |
| Uterus | Polysaccharides | | | | 9.6 \pm 2.1 |
| | Weight | | | | 633 \pm 11.0 |

¹ Four determinations on 10 to 16 glands each.

² Six determinations on 5 to 10 glands each.

³ Six determinations on 11 to 14 glands each.

⁴ Four determinations on single uterus each.

Lymnaea (Holm, '46). In contrast to other species, *Lanistes* and *Pomacea* do not have a true albumen gland. In these species the uterus is a large organ containing, at least in the case of *Lanistes*, a large amount of polysaccharide.

An egg clutch of *Lymnaea stagnalis* contained, on an average, 5.0 mg polysaccharide while the average values for the albumen gland, oothecal gland, and muciparous gland were respectively 9.9, 5.1, and 0.3 mg, totaling 15.3 mg. It appears then that the production of one egg clutch requires about 30% of the polysaccharide stored in the main glands of the female

reproductive system. The average weight of one egg clutch of *Australorbis glabratus* was 24 mg and its polysaccharide content was 0.45 mg. One albumen gland contained 2.48 mg polysaccharide; 20% of its polysaccharide is consumed during one oviposition. This evidently is a maximal figure since undoubtedly other glands also furnish polysaccharide. The detection of fucose in the polysaccharide of egg clutches but

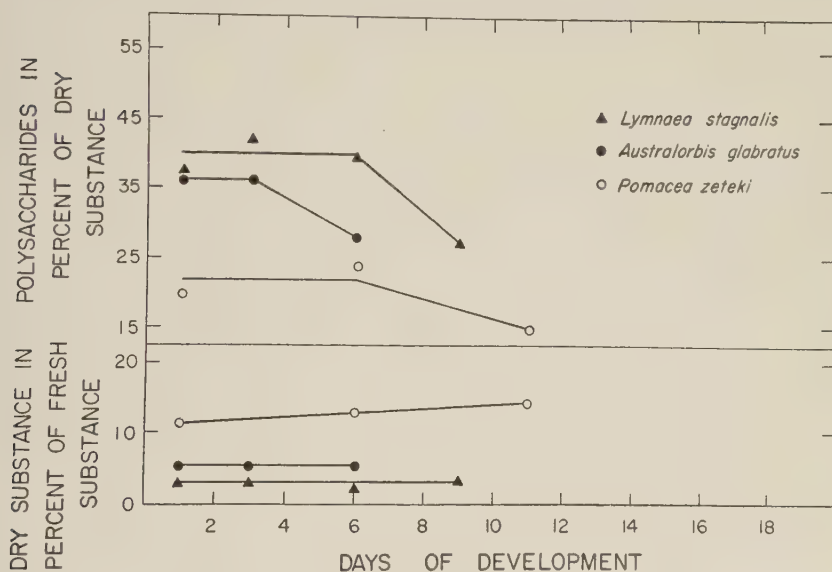


Fig. 3 Total polysaccharides and dry weights during development of eggs of aquatic snails.

not in the polysaccharide of albumen gland indicates the presence of at least one other source of polysaccharide. Glands other than the albumen gland could not be studied because they were too small for satisfactory isolation. Similar calculations were not possible with the other species because the viscous mucus of egg clutches from *Lanistes* and *Aplexa* prohibited accurate weighing and not enough specimens of *Pomacea* were available to permit quantitative study of the isolated uterus.

Figure 3 summarizes the results of quantitative determinations of dry weights and polysaccharides of egg clutches during the time of their development. The dry weight of *Australorbis* and *Lymnaea* showed little change. Apparently the amounts of organic substance metabolized by developing eggs were too small to detect. The constancy in percentage dry weight of egg clutches which had been maintained in water was rather surprising, since it might be assumed that during development some of the mucus-like material would be lost. Evidently the membrane surrounding egg clutches of *Lymnaea* or *Australorbis* is very efficient in preventing losses of material. On the other hand, the percentage dry weight of *Pomacea* egg clutches increased slightly during development. This phenomenon unquestionably is related to the fact that this species lays its egg clutches above the water line where, even in an atmosphere almost saturated with water, some evaporation must occur. Under the conditions of this experiment, such loss was not severe enough to interfere with the development of the young snails. Polysaccharides decreased noticeably in all three species only during the last days preceding hatching. As mentioned previously, eggs did not contain glycogen at the time of oviposition, but in all cases some glycogen appeared shortly before hatching. This glycogen is of course included in the total polysaccharides shown in figure 3. Therefore the galactose polysaccharides initially present decreased to a somewhat greater extent than indicated here, but the data presented in this and the foregoing section make it quite clear that a large part of the galactose polysaccharide is still present when the young snails hatch.

DISCUSSION

Glycogen was the only polysaccharide found in males of those snails (*Lanistes boltenianus* and *Pomacea zeteki*) which have separate sexes. This is not surprising since galactogen seems to be confined to the albumen gland, a structure of the female reproductive system, in the hermaphroditic *Helix pomatia* (May, '34) and *Lymnaea stagnalis* (Holm, '46).

Polysaccharide containing only galactose, that is, galactogen was obtained in this study from the albumen glands of *Australorbis glabratus* and *Lymnaea stagnalis*.⁷ The latter finding corroborates the data of Holm, ('46).

In *Lymnaea*, galactogen was the only polysaccharide demonstrable in the albumen gland, paralleling the findings with *Helix*. In contrast, the albumen gland of *Australorbis* contained some glycogen as well as a large amount of galactogen. Since no glucose polysaccharide could be found in freshly laid egg clutches of this species, it can be assumed that the glycogen of the albumen gland represents a reserve material for the energy metabolism of the glandular cells, while galactogen is essentially a secretion passed on to the eggs. A similar function probably can be ascribed to glycogen found in other glands of the female reproductive system of *Lymnaea*, the oothecal and muciparous glands. These two glands contain no genuine galactogen. In addition to glycogen, they do contain polysaccharide of the general character of mucopolysaccharides since on hydrolysis galactose, glucose, glucosamine and one unidentified sugar were obtained from the polysaccharide of the oothecal gland, and galactose, fucose, glucosamine and two unidentified reducing substances were obtained from the polysaccharide of the muciparous gland. It should be noted that polysaccharide from the oothecal gland corresponded exactly to that found in the membrane of egg clutches, while that from the muciparous gland had the same composition as the polysaccharide of mucus isolated from these clutches. It had previously been assumed on morphological grounds that the oothecal gland furnishes material for egg clutch membranes and the muciparous gland for the mucus (Holm, '46). Chemical studies reported here confirm this.

⁷ The term galactogen is used here in a general sense to indicate a polysaccharide composed entirely of galactose. We do not imply that the galactogen of *Australorbis glabratus* and *Lymnaea stagnalis* corresponds in all details to the galactogen of terrestrial snails. This is not even likely. According to our observations the galactogen of the two aquatic species has a slower electrophoretic migration than the galactogen isolated from *Otala lactea* or *Helix aspersa*, the latter two showing similar migration rates.

One other point deserves emphasis. It must be realized that the techniques used, although delicate, do not detect sugars present in extremely small amounts. This is illustrated by the observation that upon hydrolysis and chromatography of polysaccharides isolated from fresh *Lymnaea* egg clutches, no glucose was found while hydrolysis and chromatography of the polysaccharide obtained from mucus isolated from such clutches definitely demonstrated the presence of this hexose. Evidently, in the former case, the concentration of glucose was too small to be detected, having been "diluted" by the polysaccharides of the eggs proper and the membranes which do not contain glucose.

On the other hand, the absence of fucose-containing polysaccharides in newly hatched *Lymnaea* specimens probably cannot be explained on the same basis since fucose could be detected in hydrolyzates of polysaccharides isolated from adult snails where large amounts of glycogen and galactogen are present in addition to the fucose-containing compound. The polysaccharides of freshly hatched *Lymnaea* consist of approximately 70% galactogen and 30% glycogen. The fucose-containing polysaccharides probably are produced only upon sexual maturation. The large amounts of galactogen present in very young snails is rather surprising since it is confined to the albumen gland in adult specimens. According to Fraser, ('46), the first rudiment of the albumen gland appears only during the fourth week of post-embryonic life. The galactogen found in newly hatched snails is evidently a carry-over from embryonic life and must be deposited in sites other than the albumen gland.

While a true galactogen unquestionably occurs in the pulmonate snails, *Lymnaea* and *Australorbis*, it does not occur in the operculate snails, *Lanistes* and *Pomacea*. As mentioned previously, these species lack an albumen gland but have a voluminous, thick-walled uterus which stores large amounts of polysaccharide which consists of a mixture of glycogen and a galactose-fucose polysaccharide. Young egg clutches of both

species contained only the second type polysaccharide and, as in the case of some glands of the pulmonate snails, glycogen is the reserve polysaccharide for uterine tissues. The galactose-fucose polysaccharide of the uterus undoubtedly is the source of the sole polysaccharide found in freshly laid eggs. Therefore it can be assumed that in these species the uterus fulfills the role of the missing albumen gland. It must be emphasized that the galactose-fucose polysaccharides were each electrophoretically homogeneous. The migration and fucose content were not identical in both species, but the electrophoretic migration was always markedly slower than that of galactogen. Thus, there is no reason to assume that a mixture of true galactogen and another fucose-containing polysaccharide was present. It must be concluded that in these operculate snails galactogen is replaced by polysaccharides composed of galactose and fucose.

Newly laid eggs of all four species studied chromatographically contained only non-glucose polysaccharides but toward the end of their development glycogen could be detected in all of them. This observation parallels that reported by May and Weinland ('53) for *Helix pomatia* in which the glycogen of fully developed embryos represented only a small fraction of the total polysaccharide. Because of the relatively small decline in galactogen of the egg, the authors could not decide if galactogen was the source of the glycogen. The chemical studies reported here do not warrent a much more positive statement, although the following observation indicates that galactogen is probably the source of glycogen. In *Lymnaea* the proportion glycogen/galactogen rose from 0/100 at the beginning of development, to 30/70 at the time of hatching. Unfortunately, no quantitative determinations of glycogen in relation to body weight could be made with these tiny snails. Until such data are available, the possibility remains that the glucose required for synthesis of glycogen may have originated from other sources; for example, protein.

SUMMARY

1. Chromatographic and electrophoretic studies showed that galactogen occurs in pulmonate aquatic snails, but this galactogen does not correspond in all details to that found in terrestrial snails. Glycogen and polysaccharides of the general type of mucopolysaccharides were also found. The latter regularly contained galactose and fucose and in some instances glucosamine and one or two unidentified components.

2. Two species of operculate snails studied did not contain true galactogen but a galactose-fucose polysaccharide. It was found only in female snails which also contained glycogen. The latter polysaccharide alone was found in males.

3. Young egg clutches of both pulmonate and operculate aquatic snails contained only non-glycogen polysaccharides but in all species some glycogen appeared toward the end of development. Quantitative studies showed that during development some polysaccharide was consumed, but whether the galactose-containing polysaccharides are the source for the glycogen could not be determined.

4. Freshly hatched *Lymnaea* still contained large amounts of galactogen which cannot be deposited in the albumen gland as in adults because at this stage this gland is not yet formed.

5. The polysaccharides occurring in egg clutches of *Lymnaea* are derived as follows: galactogen of the eggs from the albumen gland, polysaccharides of the membranes from the oothecal gland, polysaccharides of the mucus from the muciparous gland.

6. The galactose-fucose polysaccharide found in the eggs of operculate snails is derived from the uterus which in these species fulfills the role of an albumen gland.

7. Most of the glands furnishing galactogen, the galactose-fucose polysaccharide, or the mucopolysaccharides also contained some glycogen. The latter apparently is the energy reserve for the glandular tissues, while the former are secretions utilized in the formation of the egg clutches.

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THE INDUCTION OF ARGINASE ACTIVITY IN THE CHICK EMBRYO ¹

MARTIN ROEDER

Department of Zoology, University of North Carolina, Chapel Hill, N. C.²

INTRODUCTION

Greenstein ('45) showed that in the adult rat all tissues are characterized by a definite pattern of enzymatic abilities. Spiegelman, in '48, attempted to define the origin of these patterns by supposing that in each developing cellular system a competition existed among the enzyme forming systems for available substrata, and that the successful systems occurred as a result of stabilization of the enzyme precursor when bound to the substrate. Kinetic studies of this phenomenon of enzyme induction, that is, the production by cells of particular enzyme in response to added substrate, have been made largely in micro-organisms, but Knox and Mehler ('51), Mandelstam and Yudkin ('52), and Gordon and Roder ('53) have been able to demonstrate that it occurs in such complex organisms as the rat and the chick.

Needham, Brachet, and Brown ('35) have shown that in the developing chick the system responsible for the production of urea tends to be minimized as the embryo ages, and that at least one of the enzymes necessary for the production of urea, arginase (Krebs and Henseleit, '32), decreases in activity with the change from ureotellism to uricotellism. If Spiegelman's hypothesis is valid it should be possible to reverse the direction of this change, by introducing added quantities of arginine to

¹ Portions of this paper were submitted to the Faculty of the Department of Zoology of the University of North Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

² Present Address: Department of Biology, The Woman's College of the University of North Carolina, Greensboro, N. C.

the developing embryo, and thus stabilizing the enzyme arginase, despite the natural tendency of the system to lose arginase activity. Accordingly the following experiments were carried out, with the results indicated.

METHODS

Fertilized New Hampshire Red chick eggs were secured, and incubated at 37.5°C., at constant humidity, until used. The eggs were rolled once or twice a day. Normal viability was over 95%, and hatchability over 90%.

At various times after the fifth day of incubation eggs were removed from the incubator, and solutions of various concentrations of arginine monohydrochloride (Nutritional Biochemicals) were injected into the air sac, after the technique of Gordon and Roder ('53). The fluid volume added was 0.2 ml when less than 50 mg of arginine were added, or 1.0 ml when more than that quantity was used. These volumes were chosen for convenience in making concentrated solutions, no effects of increased fluid volume were observed. At the same time control eggs were injected with like volumes of distilled water. The eggs were then sealed with paraffin, and replaced in the incubator.

After 24 or 48 hours, as indicated in the results, the eggs were removed from the incubator, the embryos freed from their membranes, and homogenized in a Potter-Elvehjem grinder, with enough ice-cold glass distilled water to bring the final tissue concentration to one part in twenty for embryos between 0.1 and 0.6 grams, to one part in ten for embryos between 0.6 and 3.2 grams, or to one part in five for larger embryos. (No effects on enzymatic activity were noticed in these ranges.) The homogenates were strained through two layers of cheesecloth, and the arginase activity measured according to the technique of Kochakian ('45) as modified by Kochakian and Vail ('47). The final production of ammonia through the action of urease was determined by aeration into 2% boric acid, and titration to the grey endpoint of methyl red-Brom cresol green mixed indicator.

The nitrogen content of both control and experimental samples were determined by the semi-micro Kjeldahl procedure (Sumner and Somers, '49).

RESULTS

The effect of adding various quantities of arginine to developing chicks is summarized in table 1. It may be seen that the addition of more than 30 milligrams of arginine is invariably

TABLE 1

The effect of the addition of varying amounts of arginine to developing chick embryos

| AMOUNT ADDED | FLUID VOLUME | CASES | SURVIVAL ¹ | INDUCTION |
|--------------|--------------|-------|-----------------------|-----------|
| <i>mg</i> | <i>ml</i> | | | |
| 250 | 1 | 8 | 0 | |
| 200 | 1 | 8 | 0 | |
| 100 | 1 | 16 | 0 | |
| 50 | 1, 0.5 | 19 | 0 | |
| 30 | 0.2 | 18 | 1 | Positive |
| 25 | 0.2 | 62 | 50 | Positive |
| 20 | 0.2 | 19 | 15 | Negative |
| 10 | 0.2 | 17 | 15 | Negative |

¹ Control eggs showed more than 90% survival in all cases.

fatal to the embryo, and that no induction of enzymatic activity can be detected when less than 20 mg of substrate is added. Accordingly the balance of the data reported, which deal with enzyme induction, will be confined to those animals which had 25 milligrams of amino acid added to the air sac.

All experimental chicks to which arginine had been added, during the period between the fifth and the fifteenth day of incubation showed an increased level of arginase activity over the normal controls. This increase ranged between 30 and 143% over the normal level of activity. Figure 1 summarizes the results of this series of measurements.

It may be seen that while normal arginase activity decreases steadily during development, after the fifth day, no such comparable decrease is observed in the level of inducible activity. Indeed this level is remarkably constant, averaging 1.43 mg of

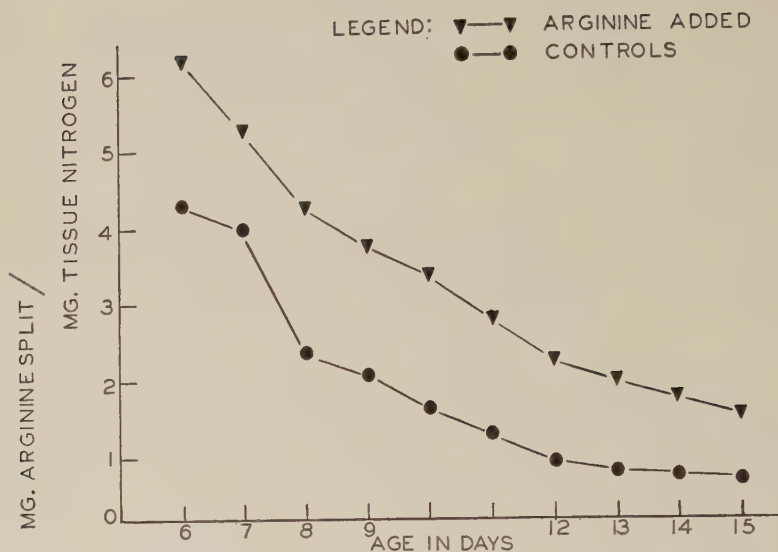


Fig. 1 The effect of the addition of twenty-five milligrams of arginine on arginase activity.

TABLE 2

Urea content of normal and experimental chicks, after injection of 30 mg of arginine to the latter

| AGE OF EMBRYO (days) | UREA CONTENT CONTROL | CASES | UREA CONTENT EXPERIMENTAL | CASES |
|-------------------------|-------------------------|-------|------------------------------|-------|
| 9 | 0.169 | 4 | 0.157 | 4 |
| 10 | 0.226 | 4 | 0.234 | 5 |
| 14 | 0.383 | 4 | 0.391 | 3 |

Values for urea content are expressed in mg ammonia nitrogen/mg tissue nitrogen.

arginine split/mg of tissue nitrogen, with a standard deviation of ± 0.36 mg. While this level varies sharply at the seventh and eighth days, it is during this period that the most profound changes occur in nitrogen excretion in this form.

In order to determine whether the sharp rise in mortality after the administration of more than 25 mg of arginine could be due to poisoning by accumulation of urea, determinations of urea content were made on dead experimental animals, and on living controls. The data presented in table 2 show no signi-

ficant differences in the amount of urea between the two groups. It must be borne in mind, however, that these determinations were made 24 hours after the addition of the amino acid. It is possible then, that in cytolysis some conversion of urea into nitrogen had occurred, although the low level of urease activity seems to preclude this as an explanation for the data. The possibility that earlier determinations might show a larger difference should not be excluded.

The level of induced activity falls to the normal level within 48 hours after the administration of arginine. These data are in accordance with those of Gordon and Roder (*op. cit.*) who observed this to be the case with induced ribonuclease activity.

If 25 mg of arginine are added twice to the same egg, the second injection being made 24 hours after the first, subsequent levels of enzyme activity are substantially the same as though only one injection had been made. The observed mortality rises, however, to 67% of the animals so treated.

DISCUSSION

The constancy which can be observed in the amount of inducible arginase activity, as opposed to the varying level of constitutive activity seems to be of special interest, for theoretical considerations of the mechanics of protein formation. As the induction of enzyme seems to be limited to a specific amount, regardless of the total enzymatic activity, it is possible that the constitutive activity represents the adjustment of the cells concerned to an overbearing environment, while the induced activity, because of its constancy, may represent the basic reserve ability of the cell to cope with sudden changes in its environment, and, because of the constancy of this amount, might better be used than the constitutive level to define, in terms of metabolic patterns, the processes of groups of cells.

In general, the entire enzyme forming system of any group of cells must be affected by the response of the cell to any change in environment, if the proteins of the cell are in equilibrium with the pool of protein forming substances. For any particular enzyme this would include the amount of formed

enzyme precursor, as well as the active enzyme. It would appear, from an extension of the data presented on arginase, that there is a limit to the amount of any one specific system which the cell may form, and exceeding this limit causes the failure of other essential systems, probably because of overly severe demands being made on the protein forming pool, to the detriment of all proteins except that one which is being induced. If this is indeed so, it would make more sense to call the amount of activity which the cell can afford to make in response to new stimuli the constitutive level of activity, as it is limited strictly by the overall enzyme pattern of the cell, while the activity which appears in control populations represents a more or less transitory adjustment, which can only be used as a yardstick within one particular environment.

Despite the radical change in metabolic patterns of nitrogen excretion, the data clearly show that the physiological ability to produce enzymes responsible for the earlier patterns is retained throughout development. In view of this it appears quite possible to test experimentally Spiegelman's hypothesis concerning the dedifferentiation of tissues by selective reversal of substrate patterns. The data presented here indicate that his thesis may be sustained by further experimentation of this type.

Since varying the level of added arginine does not result in a linear response in increased enzymatic activity, it becomes difficult to explain this induction in terms of the simple "mass action" concept of Mandelstam ('52). The shift of cellular equilibria caused by stabilization of a particular enzyme by its substrate should be linear, if no other factors are playing a role. One possible explanation which might serve to alleviate this difficulty is available. If the response to added arginine is not expressed only in the Krebs- Hensleit cycle, if indeed this response in a minor or terminal phenomenon, the data can be accommodated within the theory. Some evidence may be adduced that this is indeed the case. Brand et al. ('30) and Borsook and Dubnoff ('40) have shown that that portion of the arginine molecule which is incorporated

into urea may also be combined with glycine to form creatine. Butts and Sinnhuber ('41) demonstrated the deamination of arginine in the liver, resulting in glycogen formation. In addition Edelbacher and Koller ('34) found that the enzyme may have an activity apart from the formation of urea, in the synthesis and breakdown of nuclear protamines. If any of these alternative pathways of metabolism have a greater avidity for the substrate than does the arginine-urea system, it is quite possible that these data will conform to the mass action hypothesis.

SUMMARY

The response of the urea forming system of developing chicks to added amounts of arginine has been tested. The data show a fairly constant response, in terms of increased arginase activity, despite great changes in the constitutive level of activity. The narrow range of substrate concentration which elicits this response is discussed, and an explanation of the data is presented which attempts to fit the results into the framework of the mass action theory of enzyme formation.

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AN ANTAGONISM IN THE ACTION OF CALCIUM AND STRONTIUM IONS ON THE FROG'S HEART ¹

LYELL J. THOMAS, JR. ²

*Department of Pharmacology, Woman's Medical College,
Philadelphia, Pennsylvania*

SIX FIGURES

INTRODUCTION

The most characteristic effect of calcium on heart muscle is to promote contraction. When heart muscle is perfused with a calcium free medium, contraction does not occur but the propagated electrical response persists (Garb, '51). Furthermore, it has been demonstrated that the contractile force of heart muscle is proportional to the calcium concentration of the perfusate (Clark et al., 28). Calcium is also required for contraction in other types of muscle and seems to be a general requirement for any type of cellular movement. Thus cells will not divide and amoeboid movement will not occur in the absence of calcium. Reference to this literature can be found in the recent monograph by Heilbrunn ('56). In the case of heart muscle, the inotropic effect of calcium can be mimicked by certain other divalent cations. Among these, Ringer (1883) found that strontium acts most nearly like calcium on the frog's heart. Garb ('51) found that calcium and strontium had identical inotropic effects on the papillary muscle of the cat's heart as long as the perfusing solution contained a small amount of calcium. However, when the muscle was perfused

¹ Aided by a grant from the Penrose Fund of the American Philosophical Society.

² Present address: Department of Biology, University of Southern California, Los Angeles 7, California.

with a solution containing strontium instead of calcium the duration of the contractile and electrical responses of the muscle were greatly prolonged. The addition of a trace of calcium to the perfusate shortened the duration of these responses. Therefore it would seem that while strontium and calcium both promote contraction when used separately, calcium, paradoxically, exerts a depressant effect when present in a perfusion medium together with strontium. A similar antagonism in the action of calcium and strontium on the contractile behavior of the frog's heart was observed in this laboratory. It was believed that further study of this paradox might shed some light on the mode of action of calcium in heart muscle.

METHODS

The contractile behavior of the isolated electrically stimulated frog's heart ventricle was analyzed by the method of Hajdu and Szent Györgyi (1952) modified to permit a continuous flow of perfusate through the heart. The heart was fastened to the end of a Straub cannula by a tie around the A.V. groove thus isolating the ventricle. Contractile force was transmitted to an isometric writing lever by means of a stiff wire which passed up through the tip of the cannula and had a glass bead fastened to its lower end. The bead rested in the cavity of the ventricle. A continuous flow of perfusate from an external reservoir through the ventricle was maintained by means of a small aspirator pump powered by the oxygen-carbon dioxide aeration mixture. A slit cut in the side of the ventricle provided a rapid exit for the perfusate and an overflow hole in the cannula maintained a constant pressure head. This arrangement provided a versatile means for making rapid changes and additions in the perfusion medium without introducing artifacts into the record. A complete change of perfusate in the ventricle was effected in about 30 seconds with this arrangement. This could be judged by the fact that about 30 seconds were required for the contractile force to drop to

zero after changing the perfusate to a calcium free Ringer's solution.

Sodium and potassium analyses were done on ventricles from autonomously beating hearts which had been perfused with large volumes of solution. At the end of the perfusion period the ventricles were split in half and blotted thoroughly between two pieces of filter paper. They were dried overnight at 110°C. and weighed. Following this, the tissue was ashed at 500°C. and the ash was dissolved in a known volume of lithium chloride internal standard solution for flame photometer analysis.

The modification of Ringer's solution used in these experiments was based on data given by Boyle and Conway ('41). It contained the following millimolar (mM) concentrations: NaCl, 91.3, NaHCO₃, 12.5, KCl, 2.5, MgCl, 1.2, glucose, 32.7 and either SrCl₂, or CaCl₂ at a concentration of 1.0 mM.

RESULTS

The effect of potassium on the calcium-strontium antagonism

In confirmation of Garb's ('51) observation on papillary muscle, it was found that when the frog's heart was perfused with strontium Ringer's solution the contractions were more prolonged and forceful than normal. The addition of calcium to this perfusate depressed the force of contraction and shortened the duration of contraction. Perfusion with strontium Ringer's solution also resulted in a more rapid fatigue of the heart. Usually hearts would beat for no longer than 6 hours in strontium Ringer's solution whereas in calcium Ringer's solution they would beat for 12 hours or more. It was noticed that the behavior of hearts in strontium Ringer's solution somewhat resembled the behavior of hearts perfused with potassium free calcium Ringer's solution. In both cases the principle deviation from normal behavior appeared to be a slower rate of relaxation. Figure 1 is a diagram showing characteristic isometric contraction records (tension versus time) of an

electrically stimulated ventricle perfused with Ringer's solution containing various combinations of calcium, strontium and potassium. A comparison of B and D shows the similarity in contractile effects of strontium Ringer's solution and potassium free calcium Ringer's solution. It was further observed that the contractile behavior of hearts in strontium Ringer's solution was practically the same whether or not potassium was included in the solution (fig. 1, B and E). In

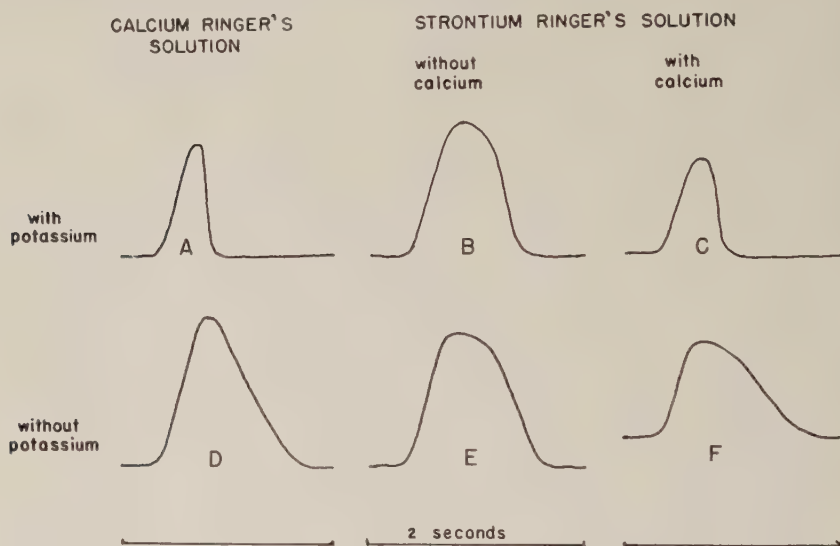


Fig. 1 Effect of various combinations of calcium, strontium, and potassium on contraction of the frog's heart ventricle (see text).

other words, frogs' hearts appeared to be rather insensitive to extracellular potassium in strontium Ringer's solution. However, the absence of potassium from strontium Ringer's solution had a profound effect on the response to added calcium. The effects of adding 0.10 mM calcium to strontium Ringer's perfusate are shown at C and F of figure 1. If potassium was present in the perfusate the addition of calcium hastened relaxation and depressed the force of contraction. If potassium was omitted from the perfusate the addition of

calcium caused a further lengthening in relaxation time and the hearts showed a tendency towards contracture. Thus the presence or absence of potassium in the perfusate seemed to govern whether calcium and strontium acted antagonistically or synergistically on the heart.

The effects of calcium and strontium on staircase

The involvement of potassium in the calcium-strontium antagonism as shown above directed the attention of the investigator to a study of the effects of these divalent cations on the staircase phenomenon. Hajdu ('53) has shown that the staircase phenomenon in the frog's heart can be correlated with net changes in the intracellular potassium content of heart muscle which occur during the contraction cycle. A negative staircase is characterized as a decrease in force of contraction when the rate of contraction is decreased. The negative staircase is presumably caused by a greater amount of potassium re-entering the muscle fiber during the interval between contractions than was lost during the preceding contraction. Hajdu found that if potassium is prevented from re-entering the fiber, by perfusion with a potassium free medium or by treatment of the heart by digitalis, the negative staircase is prevented. As shown in figure 2, calcium enhances the negative staircase when added to strontium Ringer's solution which suggests that in this case calcium enhances the uptake of potassium in the muscle fiber. However, in the light of another observation, it seems difficult to interpret the staircase phenomenon as being due solely to alterations in intracellular potassium or sodium during concentration. It was found that an increase in the concentration of calcium or strontium used in the respective Ringer's solution can produce a positive staircase. Figure 3 shows the effect on the staircase of increasing the calcium concentration in calcium Ringer's solution. At the calcium concentration normally used, which was 1.0 mM (fig. 3, A), a negative staircase resulted. When the calcium concentration was increased to 2.0 mM (fig. 3, B) the force of contraction

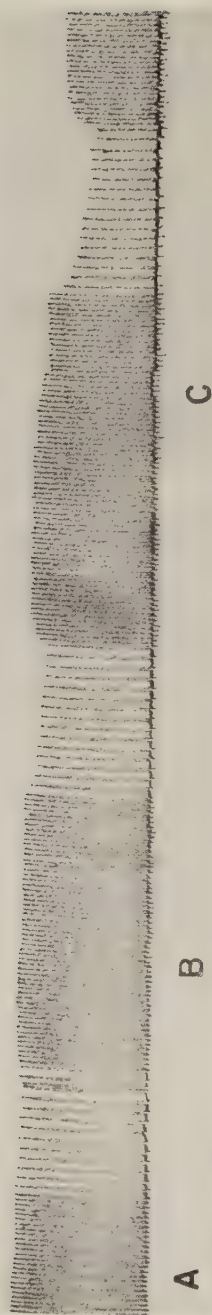


Fig. 2 Effect on staircase of adding calcium to strontium Ringer's solution. (A) Perfusate is strontium Ringer's solution containing 2.0 mM strontium but no calcium; (B) 0.04 mM calcium added; (C) An additional 0.04 mM calcium added.

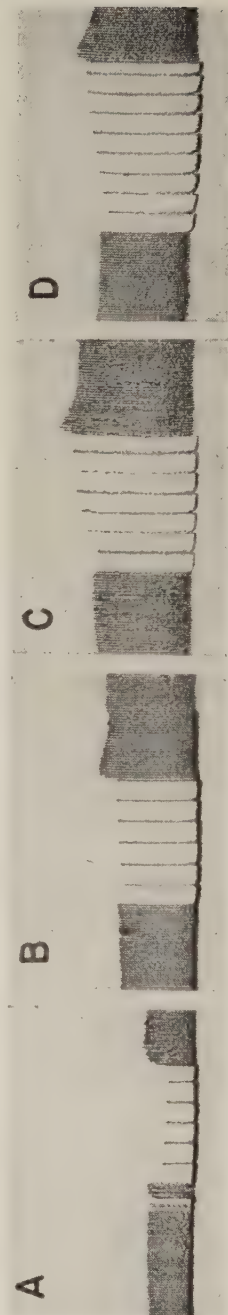


Fig. 3 Effect of calcium concentration on staircase. Ventricle perfused with calcium Ringer's solution containing: (A) 1.0 mM calcium; (B) 2.0 mM calcium; (C) 4.0 mM calcium; (D) 8.0 mM calcium.

was increased and the staircase effect was abolished. A further increase in calcium concentration to 4.0 mM (fig. 3, C) resulted in a slight additional increase in the force of contraction and also resulted in a steep positive staircase. Higher calcium concentrations than this did not appear to cause a further change in these contractile responses as shown in figure 3, D. The staircase behavior of hearts perfused with strontium Ringer's solution was found to be the same as that in calcium Ringer's solution. Thus in normal strontium Ringer's solution a negative staircase occurred which could be abolished by doubling the strontium concentration. When strontium was added to calcium Ringer's solution the effect was the same as that of adding the same amount of excess calcium. In this case the staircase was increased in the positive direction.

The effect of strontium and calcium on intracellular sodium-potassium balance

The preceding observations suggested that calcium might participate in some mechanism governing the transport of potassium in the heart muscle fiber. In particular, it seemed possible that frogs' hearts perfused with strontium Ringer's solution might be found to suffer a loss of intracellular potassium during perfusion which could be prevented by adding calcium to the perfusate. That this is the case is shown in table 1 which presents the results of a number of sodium-potassium analyses on frog heart ventricles. The unperfused controls were rinsed free of blood after removal from the frog and were then immediately prepared for analysis. All of the perfused hearts were maintained for two hours in normal calcium Ringer's solution before perfusion was started in the solutions indicated in table 1. At the end of the perfusion period all of the hearts analysed were beating autonomously. Perfusion for three hours with strontium Ringer's solution (containing the usual 2.5 mM potassium) resulted in a 30% loss of potassium from the muscle with an approximately equivalent gain in sodium as compared to the unperfused controls.

When perfusion was carried out with the same strontium Ringer's solution containing 0.2 mM calcium, the loss of intracellular potassium was decreased to about 10%. Since the contractile force of a frog's heart is greater in strontium Ringer's solution with no calcium present, the possibility that the greater loss of potassium in the former case could result from the greater contractile activity was investigated. When hearts were perfused with calcium Ringer's solution containing

TABLE 1

| MILLIMOLES OF Ca^{++} AND Sr^{++} PER LITER OF PERFUSATE | | NUMBER OF VENTRICLES ANALYZED | DURATION OF PERFUSION | MILLIMOLES OF Na^+ AND K^+ PER GRAM OF DRY TISSUE | |
|---|---------------------|-------------------------------------|--------------------------|---|------------------|
| <i>mM</i> Ca^{++} | <i>mM</i> Sr^{++} | | (hours) | <i>mM</i> K^+ | <i>mM</i> Na^+ |
| Unperfused controls | | 7 | 0 | 37 \pm 3 | 17 \pm 2 |
| 0 | 1.0 | 9 | 3 | 26 \pm 3 | 25.5 \pm 3 |
| 0.2 | 1.0 | 7 | 3 | 35.5 \pm 2 | 19 \pm 2 |
| 1.0 | 0 | 9 | 5.5 | 34 \pm 3 | 20 \pm 1 |
| 4.0 | 0 | 4 | 5.5 | 33 \pm 1 | 13.5 \pm 1 |

4 times the normal amount of calcium they contracted as forcefully as hearts perfused with normal strontium Ringer's solution. However, as shown in the last two lines of table 1, the loss of potassium (10% in 5.5 hours) from the hearts in the high calcium Ringer's solution was no greater than from hearts perfused for the same period with normal calcium Ringer's solution.

The effect of digitalis and the hypodynamic state on the calcium-strontium antagonism

As described in the section on methods, it was possible to effect rapid changes in the perfusate flowing through the heart. When the perfusate was changed from a solution containing the normal concentration of calcium to a solution containing either a higher or lower calcium concentration the heart attained a new level of contractile force within about 30 seconds. However, when the perfusate was changed from

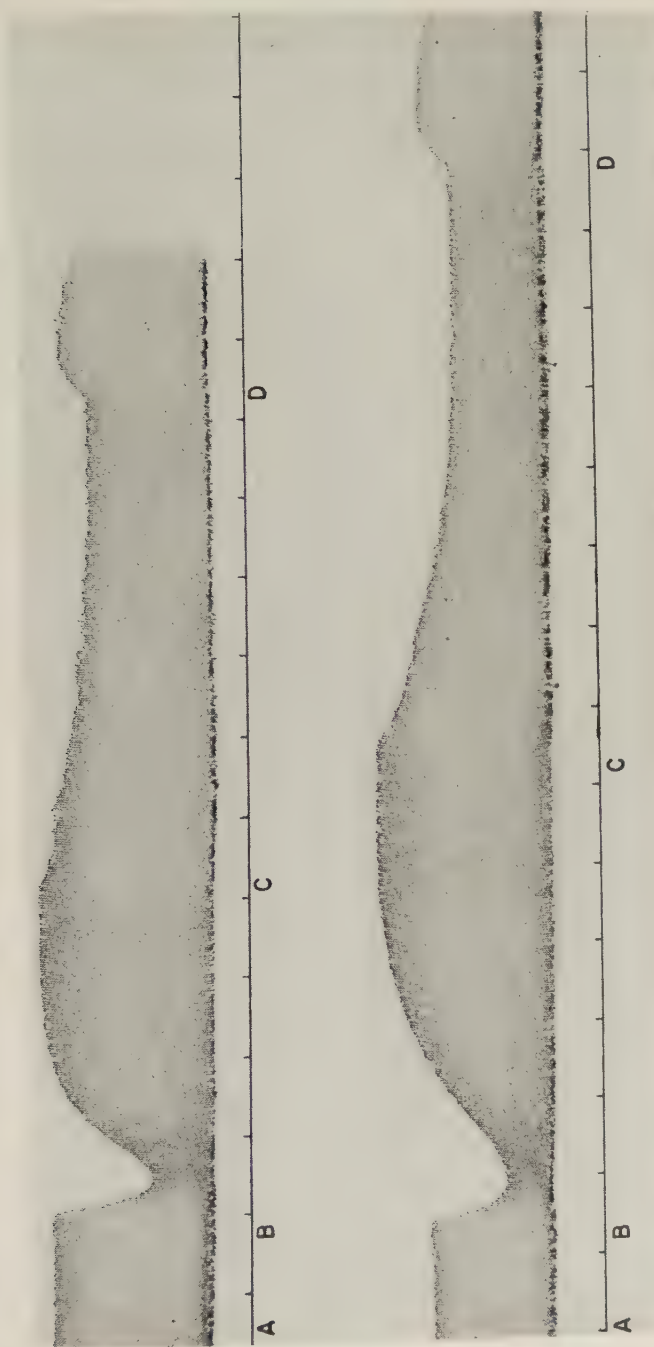


Fig. 4 Isometric contraction of ventricle stimulated at 30 times per minute. Base line time mark in minutes. Upper record from heart in fresh condition. Lower record from same heart in hypodynamic condition. In both records, (A) Perfusate is calcium Ringer's solution; (B) Perfusate changed to strontium Ringer's solution (C) 0.1 mM calcium added; (D) Perfusate changed to calcium Ringer's solution.

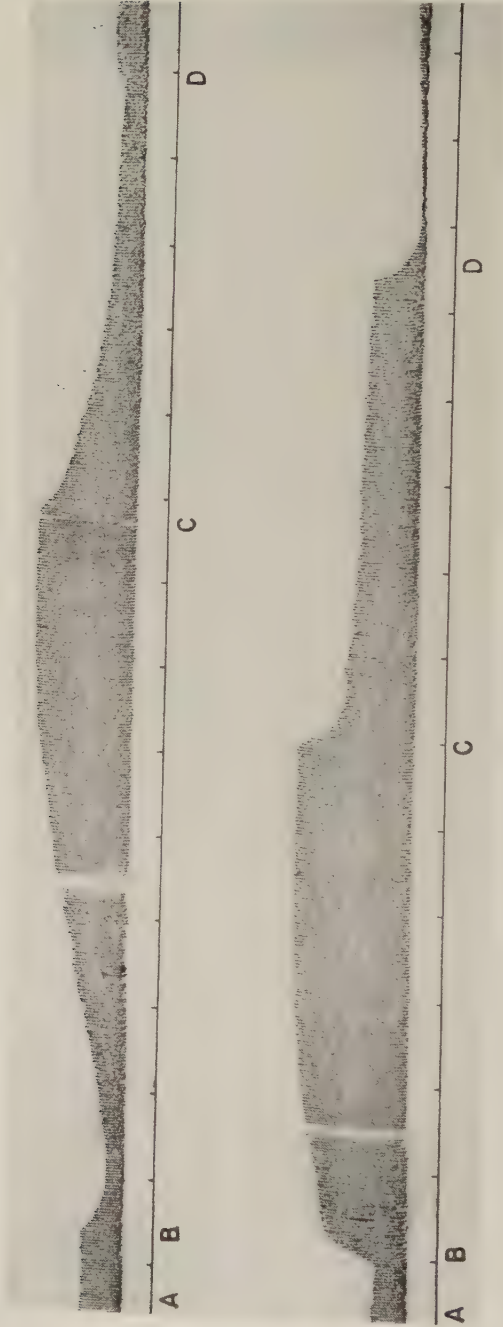


Fig. 5 Effect of digitoxin. A, B, C, and D refer to changes in the perfusate identical to those in figure 1. Upper record is from a hypodynamic heart. Lower record is from the same heart exposed to two parts per million of digitoxin for 15 minutes.

calcium Ringer's solution to strontium Ringer's solution, the attainment of a new level in the contractile force often required several minutes and the time required for this depended on the condition of the heart. This is shown in figures 4 and 5. These kymograph tracings are isometric recordings taken from ventricles stimulated at 30 times per minute. The upper record in figure 4 was taken from a fresh preparation which had been perfused for only a few minutes. The lower record in figure 4 was taken from the same preparation after it had become hypodynamic as the result of perfusion for an additional hour with calcium Ringer's solution. It can be seen that following the change to strontium Ringer's solution, at B, a longer time was required for the attainment of maximum contractile force after the preparation became hypodynamic. It will also be noted that the addition of 0.1 mM calcium, at C, resulted in a greater depression of contractile force after the preparation became hypodynamic. Figure 5 shows the effect of a large dose of digitoxin applied to a hypodynamic preparation. After making the upper tracing in figure 5 the preparation was exposed for 15 minutes to a concentration of digitoxin of 2 parts per million in calcium Ringer's solution. Note in the lower record of figure 5 that following the change to strontium Ringer's solution at B no transient depression of contractile force occurred, but instead, the force of contraction rose to a maximum level almost immediately. In fact, the change in contractile force here was as rapid as the perfusion arrangement would permit. The same applies to the speed with which the addition of 0.1 mM of calcium, at C, to the perfusate in the digitalized preparation depressed the force of contraction. Note also that the final depression of contraction by calcium (at C in fig. 5) was less after digitalization. Another characteristic effect of digitalization is shown at D in figure 5. Usually the contractile force increased after changing the perfusate back to calcium Ringer's solution. However, digitalized preparations responded with a decrease in contractile force following this change in the perfusate.

*The effect of Versene (ethylenediaminetetraacetic acid)
on the calcium-strontium antagonism*³

The effect of digitalization on the rate of change in contractile force as shown at B in figure 5 can be mimicked by adding the strontium complex of Disodium Versenate to the perfusate. This is shown in figure 6. In the lower tracing of figure 6, the perfusate was changed from calcium Ringer's solution to strontium Ringer's solution and then 0.1 mM of the strontium complex of Disodium Versenate was added as indicated. As can be seen this was followed by a rapid rate of increase in the force of contraction. It is believed that this observation is particularly significant because Versene is a che-

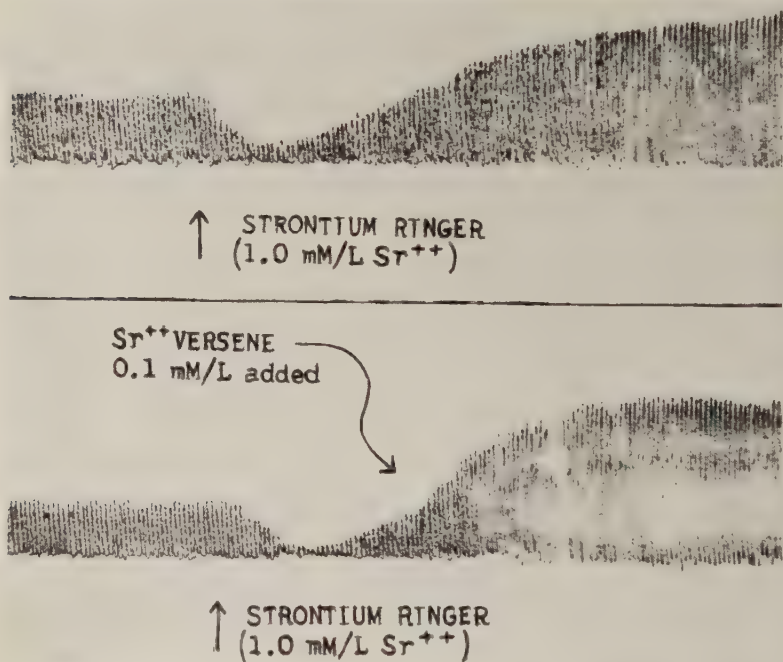


Fig. 6 Effect of strontium Disodium Versenate on the calcium-strontium antagonism (see text).

³ I wish to thank the Bersworth Chemical Company, Framingham, Massachusetts for their gift of the Versene.

lating agent which has a greater affinity for calcium than for strontium. When calcium Disodium Versenate was added either to calcium Ringer's solution or to strontium Ringer's solution, it was found to be completely without effect on the heart. Likewise, the strontium complex of Disodium Versenate had no effect on contraction when added to strontium Ringer's solution after the contractile force had reached a constant level. Therefore, the effect observed in figure 6 is, in all probability, the result of a rapid removal of calcium from the heart in exchange for strontium.

DISCUSSION

The observations on the action of Versene suggest that the calcium-strontium antagonism in the frog's heart may result from an exchange of strontium for calcium ions at some site in the muscle fiber which binds these ions in a tight complex. Further observations of a preliminary nature have indicated that this binding site is probably the cell membrane. This would be in line with the observation that calcium maintains normal sodium-potassium balance in the heart muscle when added to strontium Ringer's solution. Intracellular potassium accumulation is generally considered to be a function of the cell membrane although the mitochondria may also play a role in this process (Holland and Dunn '54). Further preliminary results have shown that Ca^{++} is bound selectively by the frog's heart in the presence of excess strontium. In these experiments frog's hearts were perfused with Ringer's solution containing 1.0 mM strontium and 0.1 mM labeled calcium. Uptake of labeled calcium by the hearts was determined by sampling the perfusate at timed intervals. The time course of calcium uptake followed a curve resembling that for contractile force depression as at C in figure 4. The strontium complex of Disodium Versenate was then added to the perfusate and another sample of the perfusate was immediately taken for counting. Subsequent samples of perfusate were taken for a period of an hour or more. It was

found that all of the labeled calcium which had been taken up by a heart was returned to the perfusate in less than two minutes. This suggests a surface binding of this ion. The rapid removal of calcium from the heart by the strontium-Versene complex also correlates quite well with the rapid contractile effect of this complex as shown in figure 6.

It was the opinion of Clark and co-workers ('28) that development of the hypodynamic state in the frog's heart is caused by an alteration in the state of the bound calcium at the heart muscle fiber surface. Loewi ('55) suggested that fluoride and oleate also exert their inotropic actions on the frog's heart by interacting with the calcium bound at the muscle fiber surface. Conceivably, the effects of digitoxin and of the hypodynamic state on the calcium-strontium antagonism as shown in figures 4 and 5 could result from an alteration in the surface of the heart muscle fiber affecting the rate of exchange of calcium and strontium ions at a binding site on the surface.

In further work an attempt will be made to correlate contractile effects like those shown in figures 4 and 5 with the rates of uptake and release by the heart of labeled calcium and strontium. A more precise study of the effects of these bivalent cations on potassium transport in the heart muscle is also planned. In addition, the problem of calcium transport into the intracellular protoplasm of the heart muscle fiber will be investigated. It seems difficult to explain the inotropic effects of calcium and strontium on contractile force and staircase as being due solely to an action of these ions on the cell membrane. On the other hand the microinjection experiment of Heilbrunn and Wiercinski ('47) suggests that contraction of muscle may be dependent upon an interaction of calcium ions with the intracellular protoplasm.

SUMMARY

1. Calcium depresses contractile force and hastens diastole in the frog's heart when added to a strontium Ringer's solution perfusing the isolated heart. This depressant effect

of calcium is contrary to the usual effect of calcium on heart muscle. When excess calcium is added to a calcium Ringer's perfusate the contractile force of the heart is increased. Strontium increases contractile force when added either to a calcium Ringer's perfusate or to a strontium Ringer's perfusate.

2. Potassium seems to be required in the strontium Ringer's perfusate for calcium to exert its depressant effect. If calcium is added to a strontium Ringer's perfusate devoid of potassium, calcium exerts an inotropic rather than a depressant effect. Conversely, potassium has very little contractile effect unless calcium is present in the strontium Ringer's perfusate.

3. When frog's hearts are perfused with strontium Ringer's solution they suffer a loss of intracellular potassium. This intracellular potassium loss can be prevented by adding calcium to the strontium Ringer's perfusate.

4. The contractile responses of continuously perfused hearts were studied during the period of transition from a strontium Ringer's perfusate to a calcium Ringer's perfusate. These observations suggest that the effects of calcium, when present in a strontium Ringer's perfusate, may be due to a release of strontium ions in exchange for calcium ions at a binding site in the muscle fiber surface. Digitoxin affects the contractile responses during the transition period in a way which suggests that digitoxin may alter the affinity of the binding site for calcium ions.

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RESTING POTENTIAL AND CONTRACTILE SYSTEM CHANGES IN MUSCLES STIMULATED BY COLD AND POTASSIUM ¹

RITA GUTTMAN, JOHN A. DOWLING AND SAMUEL M. ROSS

*Department of Biology, Brooklyn College; Harvard University Medical School;
Department of Physiology, State University of New York Medical School
and Marine Biological Laboratory, Woods Hole*

TWO FIGURES

INTRODUCTION

In a previous paper (Guttman and Gross, '56), it was demonstrated that when pretreatment of *Mytilus* smooth muscle with sea water containing excess KCl in amounts insufficient in themselves to cause contraction is followed by rapid cooling, resting potentials fall and contraction occurs. In the previous paper a threshold was established for (1) the magnitude of temperature drop necessary to achieve contraction and (2) the rate of cooling necessary for contraction. In the present paper, an attempt will be made to investigate further the role of each of the two causative factors in the phenomenon: (1) the potassium ion and (2) the cooling, in an effort to analyse some aspects of the coupling of membrane events to changes in the contractile system. Specifically, the following problems are investigated: (1) the critical amount of depolarization necessary for contraction, (2) whether the role of the K ion is a specific one or whether other ions may be substituted for it, (3) whether cooling acts by adversely affecting the metabolism and thus permitting depolarization

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and (4) whether this contraction which is brought about by a combination of chemical and thermal stimulation may be electrically relaxed by application of a sub-threshold repolarizing current.

MATERIAL AND METHODS

Electrical potentials and isotonic contractions were simultaneously recorded for the anterior byssus retractor muscle of *Mytilus edulis*. This is an invertebrate smooth muscle, the fibers of which are parallel and run the full length of the muscle (Fletcher, '36).

Large specimens of *Mytilus edulis* (about 10 cm in long diameter of the shell) were used. The anterior byssus retractor muscle which is about 4 cm. long when relaxed, was dissected out, and suspended by means of a ten gram weight in running aerated sea water before mounting.

In mounting, the muscle was threaded through holes in two rubber diaphragms, which divided a lucite chamber into three compartments (fig. 1, Guttman and Gross, '56). The portion of the muscle in one compartment was bathed in 0.26 M KCL (which contains twenty times as much potassium as is found in sea water), which served to depolarize that end (Curtis and Cole, '42). The other end of the muscle lay in a compartment filled with sea water or experimental solution. The middle inter-electrode region was bathed in paraffin oil, (Twarog, '54). Agar-sea water bridges led to beakers of sea water and 0.26 M KCL respectively, each containing a Ag-AgCl electrode.

In early experiments a galvanometer, used as a null point instrument, and a student potentiometer were used to measure potentials.

In the main bulk of the experiments, however, the potentials were amplified by a stable D. C. amplifier. The output of the D. C. amplifier was used to drive a pen amplifier, which actuated a pen motor and lever arrangement for writing on a slow kymograph. The pen motor had a response of 10 milli-

seconds. The sensitivity of the D. C. amplifier was 10 mv input for a 2 volt output.

Simultaneous recordings of mechanical and electrical changes were inscribed on the kymograph.

Fresh solutions were made up daily and pH was adjusted to that of Woods Hole sea water. Where necessary, osmotic pressure was adjusted by adding distilled water sufficient to make up a 3% salt solution.

RESULTS

Critical amount of resting potential decrease necessary for contraction. It is generally believed that contraction occurs "only when a sufficient membrane depolarization is reached," (Kuffler, '46). In an effort to determine quantitatively just how much of a change in resting potential is necessary for contraction, a cold sea water solution containing excess potassium was added to the room temperature sea water bathing the muscle, a little at a time, gradually lowering the temperature and the resting potential. If this was done slowly, it was possible to determine rather precisely at what level of potential the onset of contraction occurred. In figure 1, the resting potential in room temperature sea water was 20 mv. On gradual cooling with sea water containing seven times the usual amount of KCl, contraction occurred when the resting potential dropped to 17 mv, a change of 15%. This is a fairly typical result, the average value for seven experiments being 12%. The value was probably affected by rate of cooling and amount of excess KCl present, and it was not feasible to keep these factors constant since the thresholds of the muscles differed considerably.

Although the matter was investigated exhaustively, treating the muscle with cold sea water alone (without excess K) never resulted in contraction. This is rather surprising since cooling alone results in considerable depolarization: 10%, on the average, as compared to 12% when both cooling and

excess K are used, a difference which may not be beyond the experimental error.

In an effort to find out why cooling alone is incapable of bringing about a contraction and what the role of K may be in this phenomenon, it was decided to investigate the role of K by determining if its role is a specific one or whether other ions may be substituted for it.



Fig. 1 Critical amount of resting potential change necessary for contraction. Simultaneous recording of resting potential changes (top) and mechanical shortening (middle) of *Mytilus* muscle. Time scale (bottom) in half minutes. At (1) muscle is in sea water at 26° C, at (2) to (4) it is gradually cooled from 26° to 3° C, in sea water containing 7 times the usual amount of KCl. Contraction begins at (3) when the resting potential has dropped from 20mv at (2) to 17mv. At (4) the muscle is returned to sea water at 26° C, whereupon the resting potential rises and the muscle begins to relax.

Efforts were made to substitute the following for the excess K in the potentiating solutions: low Na, high Na, Ca, Mg and Rb. None of these was successful except for Rb.

Low Na. Following the procedures of Hodgkin and Katz, ('49), two types of low Na solutions were employed: (1) 50% choline chloride plus 50% sea water and (2) 50% dextrose and 50% sea water. Neither was an effective potentiating solution for the rapid cooling.

In this and in all subsequent procedures careful checks on the excitability of the preparation were made during and at the completion of experiments by ascertaining that the response to K remained normal.

High Na. High Na solutions were prepared by adding NaCl crystals to sea water (7.5g/1, 11g/1 and 15g/1 respectively). Unlike in the case of the high K+ solutions, no distilled water was added to correct for hypertonicity. (Adding distilled water in effect decreases the K ion concentration and these tissues are very sensitive to decreased K ions). Instead the effect of hypertonicity itself was studied by adding choline chloride in appropriate amounts to the potentiating KCl solutions. Their hypertonicity did not interfere in any way with the potentiating qualities of the high KCl solutions and so the use of hypertonic high Na solutions was felt justified.

These high Na solutions were ineffective as potentiators for rapid cooling.

High Ca. High Ca solutions (sea water plus 7 times the amount of Ca Cl₂ normally found in sea water) were prepared by adding crystals of Ca Cl₂ and again not correcting for hypertonicity. This procedure was followed as mentioned above because the tissue is believed to be more sensitive to decreased K than to hypertonicity. High Ca solutions also proved ineffective as potentiators.

High Mg. High Mg solutions (sea water plus 7 times the amount of Mg Cl₂ normally found in sea water) were prepared by adding crystals of Mg Cl₂ to sea water and again

not correcting for hypertonicity. These high Mg solutions also proved ineffective as potentiators for rapid cooling.

Rb. RbCl was added to sea water and proved very effective as a potentiator for rapid cooling. Indeed it played a potentiating role in concentrations far lower than in the case of KCl.

That Rb should be more effective than K is not surprising for while the atomic size of Rb exceeds K, its hydration shell is smaller² and it has a higher mobility.

It was estimated that there are about 13 mM of KCl in Woods Hole sea water (Curtis and Cole, '42). When the amount of KCl added to sea water was between 3 and 7 times this amount, it caused no contraction in itself but was effective as a potentiator for rapid cooling. In the case of RbCl, 13mM RbCl added to sea water proved an effective potentiator. Indeed, $6 \times 13\text{mM}$ of RbCl, or 78 mM RbCl, resulted in so violent a contraction on rapid cooling that the muscle recovered only slightly and then became irreversibly inexcitable.

Sandow and Mandel ('51) also found that Rb depolarizes frog sartorius muscle, as might be expected from its chemical similarity to K.

This leads us then to the next question. What is the role of the rapid cooling? Does cold "liquify" the membrane, as Heilbrunn believes? Heilbrunn ('56) holds that the cortex contains both lipid and protein, both combined with calcium and that it is the calcium that keeps the cortex rigid. He assumes that some of the calcium is bound directly to protein and that a drop in temperature would free this calcium since the cation-binding power of a protein decreases with a decrease in temperature (Austin, Sunderman and 'amaack, '27). Heilbrunn points out that "stimulating agents such as heat, cold and KCl, which at first glance have little

² Conway ('47) states that the hydrated ionic size of Rb and K are almost identical (i.e. 3.6 and 3.8 Å respectively), while Na has a much greater hydrated ionic size (i.e. 5.6 Å). Our results, viz. that Rb but not Na can be substituted for K in these experiments are consistent with Conway's data.

in common and could scarcely be expected to produce the same effect on protoplasm, actually do, all of them, cause a release of Ca from homogenized muscle fibers of the frog (Weimar, '53).''

Or does the cold perhaps adversely affect the metabolic processes which normally prevent K leakage across the membrane and does it thus permit contraction? To investigate this problem the muscle was treated with N₂, Na iodoacetate and NaCN respectively.

Anaerobiosis and metabolic poisons. Muscles were bathed in sea water which had been boiled and through which nitrogen gas was bubbled for 2 1/2, 3, 4 1/2 and 5 hours respectively. In some cases the anaerobiosis technique of Benesch and Benesch ('53) in which O₂ is removed from the solution by the glucose-glucose oxidase, catalase system was utilized in addition. Despite all these precautions in removing traces of O₂ from the system and despite the length of treatment, there was no inhibitive effect and the phenomenon which we have been describing persisted.

Treatment with 0.001M NaCN and 0.001M NaIAc for long periods also did not inhibit the phenomenon.

In conclusion, then, we were unable to demonstrate that the phenomenon is metabolism-dependent.

Application of sub-threshold repolarizing currents

It was felt that it might be of some interest to ascertain whether these contractions which are caused by chemical and thermal stimulation could be relaxed electrically by imposing an anodal electrotonus on the experimental end of the muscle. This was indeed possible. In figure 2, a sub-threshold repolarizing current of 100 μ A was repeatedly applied by placing on the muscle wicks moistened with sea water and leading off to silver-silver chloride electrodes. There is distinct relaxation of the muscle each time the repolarizing current is applied, when the anode is on the experimental end of the muscle. Why the relaxing effect should diminish with each application of the anodal current

is not clear. It may be for non-physiological reasons, e. g. injury by the rather strong repolarizing current applied for a relatively long time, or else it may be that after one relaxation subsequent repolarizing current applications have a lesser degree of contraction to counteract and so the effect is less. Be that as it may, it is interesting that an electrotonus can apparently relax a muscle irrespective of how the contraction was produced: electrically, chemically or thermally, (cf. Fleckenstein, Hille and Adam, '51).

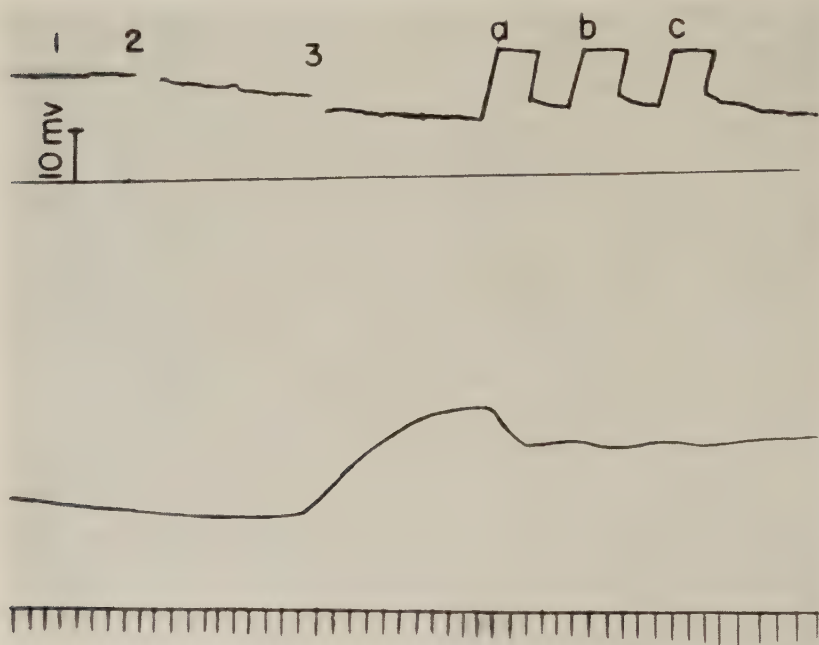


Fig. 2 Effect of D. C. repolarizing currents. Simultaneous recording of resting potential changes (top) and mechanical shortening (middle) of *Mytilus* muscle. Time scale (bottom) in half minutes. At (1) muscle is in sea water at 24° C.; at (2) it is prepotentiated with 25° C. sea water containing 6 times the usual amount of KCl; at (3) it is rapidly cooled with 3° C. sea water containing 6 times the usual amount of KCl, and the muscle contracts. A D. C. repolarizing current of 100 μ A is applied with the anode on the experimental end of the muscle at (a), at (b) and again at (c), allowing equal time intervals between. It can be seen that application of the anode to the experimental end of the muscle causes marked relaxation.

DISCUSSION

Recent work on the anterior byssus retractor muscle of *Mytilus edulis* utilizing A. C. methods (Schmandt and Sleator, '55; Hoyle and Lowy, '56) has tended to clarify some of the hitherto puzzling aspects of the physiology of this muscle. Much of the irregularity of its electrical activity is now ascribed to its complex innervation: the presence of ganglion cells in the muscle and the lack of uniformity in the innervation of different parts of the muscle (Bowden and Lowy, '55).

For our purposes, however, the preparation proved valuable since it generally showed clear cut responses to rapid cooling and remained excitable for many hours. Also, it is a parallel fibered muscle and the fibers run the full length of the muscle, so that electrically it acts like one fiber, (Fletcher, '36). Most important of all, the extreme slowness of the contractions favors analysis of the events occurring in the muscle upon stimulation.

Indeed, this muscle is so extremely slow (sometimes as much as 45 minutes is required for contraction and an even longer time for relaxation) that it was possible in effect to extend the time scale and demonstrate in "slow motion" what occurs during depolarization and subsequent muscular activity when a muscle is stimulated by chemical and thermal means. This slow time course made it possible, for instance, to determine rather precisely the critical amount of depolarization necessary for contraction when a muscle is gradually cooled.

That Rb may be successfully substituted for K as a "potentiator" so that subsequent cooling is effective is of interest. Gallego and Lorente de Nó ('47) and also Feng and Liu ('49) produced depolarization of nerve with RbCl and took the view that this depolarization cannot be explained on the basis of the Bernstein theory. The latter workers were indeed able to show that the usual linear relationship holds for Rb concentrations. They claimed that since there is no Rb inside the membrane, the potential cannot depend

on "the concentration ratio of Rb inside and outside" and they thus reject the Bernstein theory of the resting potential. Sandow and Mandel ('51) argue, however, that these investigators miss the essential feature of the Bernstein hypothesis, i. e. that the membrane potential is a *diffusion* potential. They argue that in a normal cell the membrane potential may be due to a potassium diffusion potential and its magnitude will then vary linearly with the log $K_{\text{inside}} / K_{\text{outside}}$ but that if the external KCl is replaced by RbCl, it is "still possible to have another diffusion potential set up at the membrane, of the type referred to as a 'chemical' diffusion potential and its magnitude will vary somehow with the external concentration of Rb". Sandow and Mandel found experimentally that the membrane potential does indeed vary linearly with the log of the external Rb ion concentration in frog sartorius muscle, and that the Henderson equation for the potential

$$E = 58 \log \frac{U_K K_{\text{in}}}{U_{\text{Rb}} \text{Rb}_{\text{out}}} \text{ mV}$$

where U_K and U_{Rb} represent respectively the mobilities of K^+ and Rb^+ , holds. Our results with *Mytilus* smooth muscle, where Rb may be successfully substituted for K as a "potentiator" so that subsequent cooling is effective, probably represents another system similar to that discussed by Sandow and Mandel.

Perhaps the most interesting findings were those obtained with the use of the anodal repolarizing currents, since these throw light upon the probable mechanism involved when the muscle is caused to contract by treatment with excess K and rapid cooling. The data indicate that if the membrane is repolarized by means of anodal currents, the contracted muscle relaxes. This suggests that cooling and excess K cause contraction specifically because they result in depolarization.

Throughout, the term, "contraction," has been used in a general sense, i. e. not in the sense of a mechanical change such as a twitch or a tetanus accompanied by an action

potential. The results obtained with anodal currents suggest strongly, however, that in these experiments with excess K and rapid cooling, we are dealing specifically with prolonged depolarizations resulting in contractures. (Contractures may be defined as contractile responses that are prolonged, reversible and non-propagated, [Gasser, '30]).

SUMMARY

Simultaneous recordings of mechanical changes and resting potential changes in *Mytilus* smooth muscle on stimulation by thermal and chemical means were made in an effort to investigate the general problem of the coupling of membrane events to changes in the contractile system.

1. Cooling alone never results in contraction, although it does cause a marked decrease in resting potential. If the muscle is bathed in sea water containing subthreshold amounts of excess K and then cooled, however, a decrease in resting potential occurs and the muscle contracts.

2. The average critical amount of resting potential decrease necessary for contraction obtained on gradual cooling with excess K amounts to about 12% of the original value.

3. Rb (in even lower concentrations than K) may be substituted for K in this phenomenon.

4. That the phenomenon is probably not metabolism-dependent was determined by experiments with metabolic poisons.

5. It was found possible to relax a muscle electrically by application of an anodal current to the experimental end, after it had been stimulated by thermal and chemical means.

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COMPARATIVE HISTOCHEMISTRY OF SELECTED TISSUES FROM ACTIVE AND HIBERNATING ARCTIC GROUND SQUIRRELS, SPERMO- PHILUS UNDULATUS¹

WILLIAM V. MAYER² AND SOL BERNICK

*Department of Biology, Wayne State University, Detroit 2, Michigan, and
Department of Biology, University of Southern California,
Los Angeles 7, California*

TWELVE FIGURES

INTRODUCTION

Using the periodic acid-Schiff reaction (Lillie, '54), Lyman and Leduc ('53) demonstrated the concentration of glycogen in the liver, heart, and skeletal muscle of hibernating and active hamsters. As pointed out by Lyman and Leduc ('53) there is little agreement in the literature as to the comparative amounts of glycogen in the tissues of warm and active and hibernating animals. The present investigation, using the Arctic ground squirrel, *Spermophilus undulatus*, was undertaken to ascertain the concentrations of glycogen in the heart, skeletal muscle, and liver of an additional species of hibernator comparing both the hibernating and the warm and active states. In addition, modern histochemical techniques have made it possible not only to determine the presence of glycogen in tissues but to detect concentrations of lipids and alpha-amino acids as well. In order to indicate possible comparative energy sources in the hibernating and active squirrels these tests were also utilized. As far as is

¹ This research was supported by the United States Air Force under Contract No. AF 18 (600) - 843, monitored by the Arctic Aeromedical Laboratory, Alaskan Air Command, Ladd Air Force Base, Alaska.

² Present address: Department of Biology, Wayne State University, Detroit 2, Michigan.

known no previous work of this type has been accomplished using the Arctic ground squirrel as an experimental animal.

MATERIAL AND METHODS

Tissues from eight Arctic ground squirrels were utilized for these experiments as shown in table 1. Of the four warm and active animals three had been awake for a minimum of 14 weeks prior to sacrifice. The fourth animal had been awakened from hibernation and sacrificed when fully awake. No temperature is reported for this animal as it was highly

TABLE 1

*Vital statistics on the eight Arctic ground squirrels, *Spermophilus undulatus*, used in these experiments*

| NUMBER | SEX | CONDITION | WEIGHT IN GRAMS | TEMPERATURE °C |
|--------|--------|--|--------------------|-------------------|
| 82 | male | warm and active | 776 | 37.8 |
| 70 | male | warm and active | 1024 | 35 |
| 6 | female | warm and active | 617 | 35 |
| 62 | female | awakened from hibernation and killed | 839 | .. |
| 63 | male | hibernating | 1087 | 3 |
| 43 | male | supercooled in hibernation | 940 | -4.6 |
| 77 | male | died of starvation in hibernation | 262 | 11.2 |
| 78 | female | hibernating | 586 | 11 |

variable through the period of arousal. The numbers used are those by which the animals were identified in the animal colony of the University of Southern California. Two of the four animals killed in hibernation were subjected to additional stresses. One, number 43, was placed in a $-15^{\circ}\text{C}.$ temperature at 1245. By 1600 the previously inserted rectal thermocouple indicated a body temperature of $0^{\circ}\text{C}.$ and by 1900 the supercooled temperature of $-4.6^{\circ}\text{C}.$ was attained. At 1908 there was an immediate sharp rise in the temperature to $0^{\circ}\text{C}.,$

indicative of the formation of ice within the body (Kalabuchow '35). Tissues were removed from this animal after death. A second animal, number 77, had been exposed to -15°C . temperatures for 45 days without food or water. During this period the initial weight of 667 grams dropped to 262 grams and tissues were taken from the squirrel as soon as it was discovered to be dead.

The warm and active animals were killed by squeezing the thoracic region to mitigate against the possible effects of anaesthetics on the tissues. Other mechanical methods of killing were not employed due to the necessity of recovering undamaged skeletal material from the cadavers. Except for the starved and supercooled animals, which died in the process of experimentation, the hibernating squirrels had the tissues removed without anaesthesia, as the process of hibernation itself provided adequate torpor for such surgery.

Samples of liver, tongue, gluteus maximus muscle, pancreas, spleen, and heart were removed from each animal and pieces approximating 3 millimeter cubes were fixed in 10 per cent formalin, alcoholic formalin, Helly's fluid, and modified picro-acetic-formalin. The fixed tissues were dehydrated and prepared for imbedding in both paraffin and nitrocellulose in the routine manner. The tissues were sectioned at thicknesses varying from 8 to 25 micra and stained with hematoxylin and triosin for routine histological examination. For the purposes of this report only the liver, tongue, and heart were subjected to further histochemical treatment.

The ninhydrin-Schiff reaction (Lillie, '54) was utilized to demonstrate the presence of alpha-amino acids in the liver and tongue muscle of two animals in hibernation, numbers 63 and 77, and two warm and active squirrels, numbers 82 and 6.

The investigation of tissue lipids was undertaken using tissues which were frozen, sectioned, and stained with Sudan black B (Lillie, '54). The tissues were taken from hibernators, numbers 77, 78, and 43 and from warm and active animals numbers 6 and 82.

The livers and tongues of all eight animals were subjected to the periodic acid-Schiff reaction to ascertain glycogen content. The heart muscle from hibernators numbers 63 and 43 and from warm and active animals numbers 6 and 62 was likewise subjected to the periodic acid-Schiff reaction to determine its glycogen content.

Four or five slides were prepared of the tissues of each animal under investigation and examined under high magnification for significant detail.

RESULTS

The use of the ninhydrin-Schiff reaction for the demonstration of alpha-amino acids in the liver gave the results as noted in figure 1. Figure 1A shows the typical liver section from a hibernating squirrel in which there is an absence of clearly demonstrable alpha-amino acids. Figure 1B indicates the typical picture of the liver of a warm and active squirrel in which there is an abundance of alpha-amino acids as would be expected from normal protein hydrolysis. A similar absence of alpha-amino acids is noted in the tongue muscle of the hibernators as shown in figure 2A, while the warm and active animal demonstrates protein degradation taking place in the muscle as illustrated in figure 2B.

The lipid content of the livers of those animals which were warm and awake was negligible as shown in the section stained with Sudan black B (fig. 10). The livers of the animals in hibernation, however, showed definite fat droplets in the cytoplasm of the parenchymal cells of the liver (fig. 11). The supercooled animal, number 43, showed even a more dramatic concentration of fat in the liver with the fatty degeneration of the organ quite pronounced (fig. 12). On staining with haematoxylin and triosin large intra- and extracellular fat-like vacuoles were pronounced. In the sections treated with Sudan black B (fig. 12) large fat globules are present in and around the liver cells.

The liver sections of animals in hibernation treated for the presence of glycogen gave the appearance shown in

figure 5 except for that of animal number 43. Glycogen can be seen to be sparsely and unevenly distributed in the livers of these cold and torpid animals. An intermediate state of contained glycogen is noted in figure 4 which represents a liver section of the squirrel awakened from hibernation and then sacrificed. More glycogen is present in the region labelled "a" than in comparable regions from the livers of hibernating animals. Region "b" approximates the concentration of liver glycogen noted in the livers of hibernating squirrels, and region "c" is similar in glycogen concentration to the depleted areas in the liver sections from hibernating squirrels. Figure 3 shows the glycogen content of the typical liver of the warm and active ground squirrel. The one supercooled animal displayed the heaviest concentration of glycogen of any of the animals in this study as shown in figure 6. This animal showed microscopic evidence of fatty degeneration. It appears that relocalization of the glycogen occurred in the liver. As the hepatic cells toward the central vein degenerate with the deposition of fat, the glycogen is stored in the uninvolved peripheral cells.

The muscle glycogen concentration follows the pattern of that of the liver glycogen rather closely. In hibernating squirrels the tongue muscle demonstrates a dearth of contained glycogen as indicated by figure 9 in which the sparseness of the contained glycogen allows for the observation of the cross striations of the muscle fibers. In the animal awakened from hibernation and then killed, shown in figure 8, the tongue muscle glycogen store is greater than that of the hibernator, but still does not approximate the glycogen content of the tongue muscle of the fully awake and active squirrel shown in figure 7. In figure 8 cross striations are still detectable in the muscle, but in the tongues of warm and active squirrels they are obscured by the presence of glycogen which can be seen to have migrated to one side of the cell in the cross sections shown, possibly a fixation artifact.

The pattern of contained glycogen in the heart is different from that of the tongue or liver in that those animals in

hibernation showed the presence of glycogen in regional, banded concentrations within the muscle cells. In the warm and active animals this pattern of glycogen was not noted and the heart muscle did not appear to contain any glycogen.

DISCUSSION

The absence of appreciable alpha-amino acids in the livers of hibernating squirrels indicates a low rate of protein metabolism in the hibernator. That digestion has stopped and no alpha-amino acids are coming from the digestive tract does not mean that there is no hydrolysis of protein taking place. It is possible that protein degradation is taking place at such a low rate as to have the resultant products used almost as rapidly as they are produced and thus leave none to accumulate in the liver of the hibernating animal. There is also the possibility that the reduction and, perhaps, cessation of protein metabolism may simply be due to the lessened body temperature of the hibernator. At lowered body temperatures it is possible to postulate that the reactions incident to protein catabolism may not be able to proceed. Haurowitz ('50) has mentioned a reduction in the rate of denaturation of protein solutions by storage at low temperatures, perhaps indicative of reduction of other types of protein metabolism at lowered temperatures.

The muscle alpha-amino content likewise follows the pattern of that of the liver in showing negligible protein degradation in the muscle cells of hibernators, while the presence of the alpha-amino acids in the muscle cells of the warm and awake squirrels is indicative of a greater protein catabolism in these animals.

Although adequate stores of glycogen are to be found in the tongue muscles and the livers of the non-hibernating squirrels the glycogen content of these tissues in the hibernating squirrel is markedly decreased. This appears to be due to the fact that the energy for the maintenance of metabolism would come first from the glycogen stores of the body. Even though metabolism in the hibernator is at a low level it is

still apparent that even the glycogen stores of the muscles which are inactive are utilized during hibernation for the maintenance of muscle metabolism. It is also obvious that the length of time the animal is in hibernation will determine the point to which the glycogen levels of the tissues will drop. It has been observed in earlier experimentation by the senior author that the periods of torpor did not last longer than three weeks, with a two week period of hibernation without arousal being more the normal condition. This timing of the periods of arousal during hibernation may be due to exhaustion of the readily available energy sources in the tissues. It is then necessary for the animal to either awaken and eat, and the senior author has noted the storage of food in the hibernating nests by Arctic ground squirrels (Mayer, '53), or to convert greater amounts of fat at higher body temperature into energy sources available for the maintenance of life at hibernating temperatures.

On a hibernating animal with a body temperature of 3°C., number 63, opened in the cold room it was noted that the heart beat 11 times per minute while but one breath was taken in this same time interval. The demands on the heart muscle, then, seem the greatest for any of the muscles of the body during hibernation. In light of this it is interesting to note that even when liver and tongue muscle showed a depletion of glycogen reserves the heart muscle contained localized bands of glycogen. These bands of glycogen evidently supplied the energy during hibernation to keep the heart beating and, as noted by Lyman and Chatfield ('55), to allow the heart to increase its work load during the early stages of arousal without depending on any exogenous source for energy. The heart muscle of the warm and active animal showed no concentration of glycogen in bands, and little glycogen in any form.

With the exception of the supercooled animal the livers of those squirrels in hibernation showed a decrease in the amount of glycogen over that noted in the livers of the warm and active animals. This is at variance with the results of

Lyman and Leduc ('53) who noted little difference in glycogen content between the livers of hibernating and non-hibernating hamsters. That the liver is not an essential organ in providing glycogen as an energy source for the awakening hamster is indicated in the work of Lyman and Leduc ('53) who cut off the blood supply to the liver by evisceration and ligation and still had the rostral portion of the hamster raise to a temperature of 37°C. without the availability of the liver glycogen. Figures 3, 4, and 5 form a graded series showing lessened amounts of liver glycogen from the warm and awake to the recently awakened and finally, the hibernating squirrel. The fact that the recently awakened animal shows more liver glycogen than the hibernating one can only be explained by a process of glycogenesis using some other body source of energy. As protein in hibernators is metabolizing slowly it is apparent that lipids are the only other readily available energy source for arousal.

The liver of the supercooled animal is most heavily invested with glycogen. The cause for this may have to be looked for outside the liver. The pancreas may have failed to function properly. An insulin deficiency would account for the failure to utilize stored glycogen and the failure of the pancreas to produce choline may account for the failure of lipid metabolism and the subsequent fatty degeneration of the liver. Fatty degeneration of the liver is sometimes due to excessive protein oxidation, but the low level of protein metabolism in the livers of the hibernating squirrels would rule this out as a source for the fatty liver.

From the tissues examined it is apparent that the available glycogen stores are utilized during hibernation in the maintenance of metabolism. This fact, coupled with the small amount of demonstrated protein metabolism, leaves only the lipids of the body to act as an energy reserve either directly or through a process of glycogenesis. While the liver is not normally mentioned as a fat depot in warm and active animals, this condition apparently changes with the onset

of hibernation. The livers of the active animals showed a negative sudanophilia as pictured in figure 10, while the livers of the squirrels in hibernation showed the presence of fat droplets as indicated in figure 11. With the exception of the supercooled animal discussed above, the liver lipids and glycogen are present in inverse proportion to one another. The lessened liver glycogen and the presence of lipids strongly suggests the metabolism of lipids to account for the energy of awakening from hibernation. The work of Fawcett and Lyman ('54) has already indicated the lowering of the iodine value of the lipids of hibernating hamsters in response to low environmental temperatures which would make the lipids a more readily available energy source for the hibernator.

Lyman and Chatfield ('55) state that it is generally agreed that glycogen is the source of energy during arousal. However, Pembrey ('01) reported respiratory quotients as low as .295 in hibernating marmots and postulated that not only was fat being utilized as an energy source but that it was also being converted to glycogen. Benedict and Lee ('38) also noted that the respiratory quotient for the hibernating marmot was consistently less than .83 and came to the conclusion that the energy for arousal was derived from lipids. The findings of this study would seem to favor these latter views and, it is hoped, may instigate further investigations of the role of lipids as energy sources in hibernators.

SUMMARY

Liver, heart, and tongue muscle of hibernating and non-hibernating Arctic ground squirrels, *Spermophilus undulatus*, were examined for glycogen, alpha-amino acids, and lipids by histochemical techniques.

In muscle and liver the degradation of proteins during hibernation was negligible, perhaps due to the low body temperature of the hibernators. In warm and active squirrels the liver and tongue muscle showed positive reactions for alpha-amino acids.

The liver and muscle glycogen content in hibernators was lower than that in the livers and muscles of warm and awake animals. The reverse situation was true for the heart muscle.

Lipids were noted to be in greater concentration in the livers of hibernating squirrels than in livers of the non-hibernators.

The results indicate the need for further investigation of lipids in the process of arousal from hibernation of the Arctic ground squirrel.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

- 1 A Liver of hibernating Arctic ground squirrel stained for alpha-amino acids by the ninhydrin-Schiff method. Note the typical absence of alpha-amino acids characteristic of hibernating squirrels. (90 X)
- 1 B Liver of warm and awake Arctic ground squirrel showing the positive reaction to staining by the ninhydrin-Schiff technique for the presence of alpha-amino acids. Sections 1 A and 1 B are mounted on the same slide and treated in exactly the same fashion. (90 X)
- 2 A Striated muscle from the tongue of a typically hibernating Arctic ground squirrel treated by the ninhydrin-Schiff technique for alpha-amino acids. The reaction is similar to that demonstrated in figure 1 A. (90 X)
- 2 B Striated muscle from the tongue of a typically warm and awake Arctic ground squirrel treated with the ninhydrin-Schiff technique. This section shows the positive reaction for alpha-amino acids as did the liver indicated in figure 1 B. Sections 2 A and 2 B are mounted on the same slide and treated in exactly similar fashion. (90 X)
- 3 Liver section from a typically warm and active ground squirrel stained by the periodic acid-Schiff technique. The hepatic cells show a dense deposition of intracellular glycogen granules. V = interlobular vein. (440 X)
- 4 Liver section from an Arctic ground squirrel awakened from hibernation and sacrificed. Stained by the periodic acid-Schiff technique for the demonstration of glycogen. a, b, and c are regions of different density of glycogen deposition. V = interlobular vein. (440 X)
- 5 Liver section from a squirrel killed in hibernation. Section stained by the periodic acid-Schiff technique. Note the decreased quantities of glycogen in the parenchymal cells. V = interlobular vein. (440 X)
- 6 Liver section from squirrel supercooled to -4.6°C . stained by the periodic acid-Schiff technique. This liver showed a fatty degeneration. The glycogen is deposited at the peripheral portion of the liver lobule. V = interlobular vein. F. I. = fatty infiltration. (440 X)

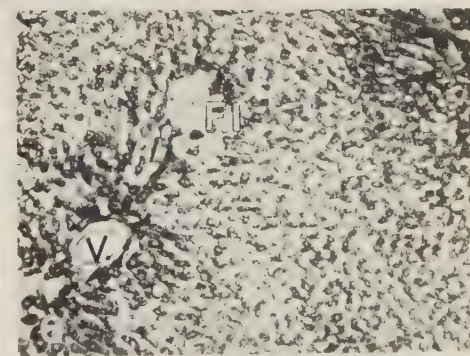
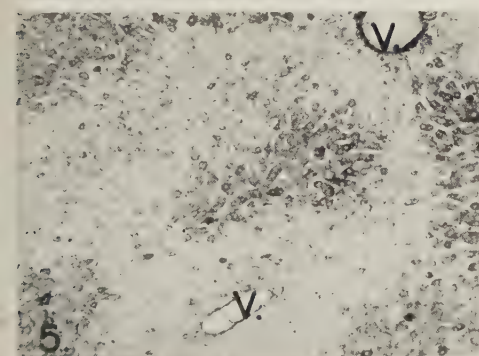
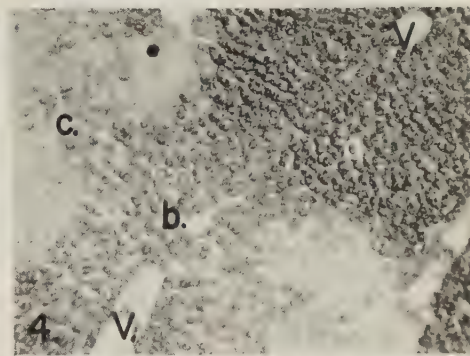
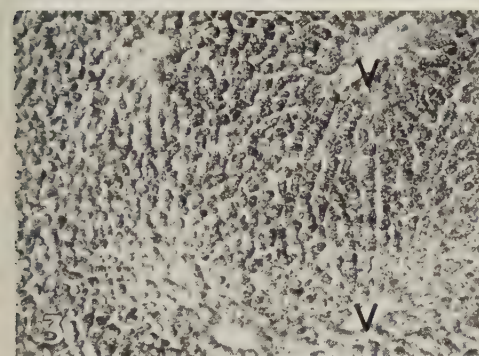
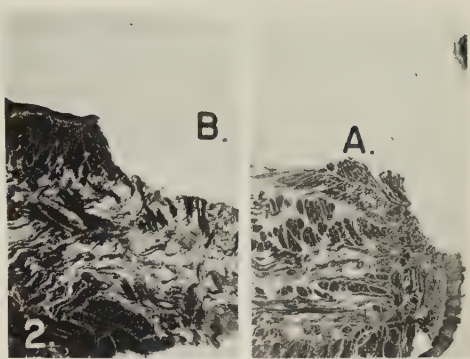
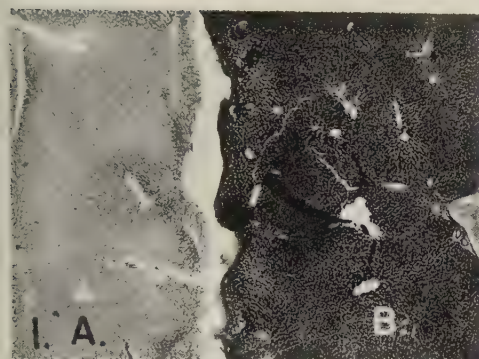
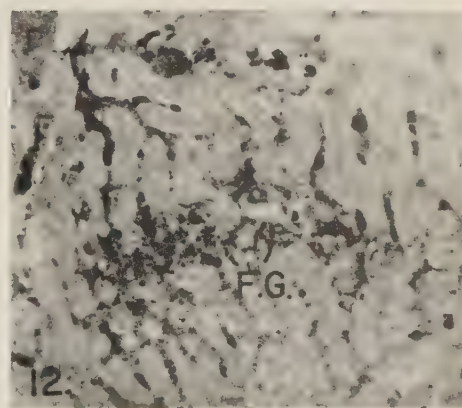
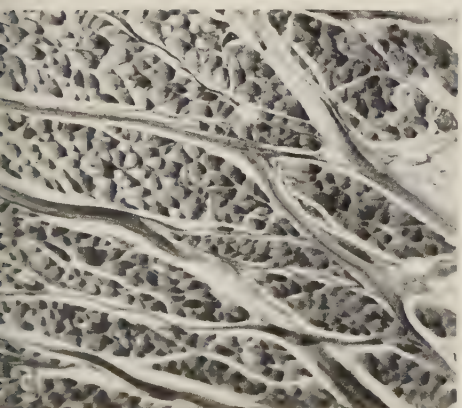
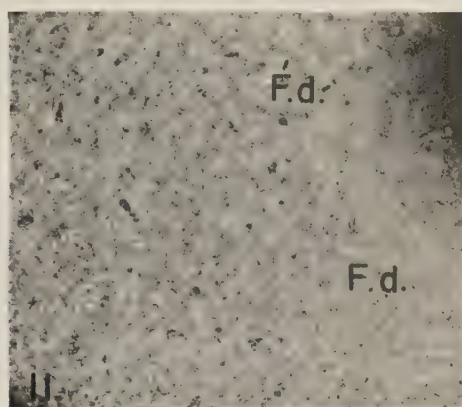
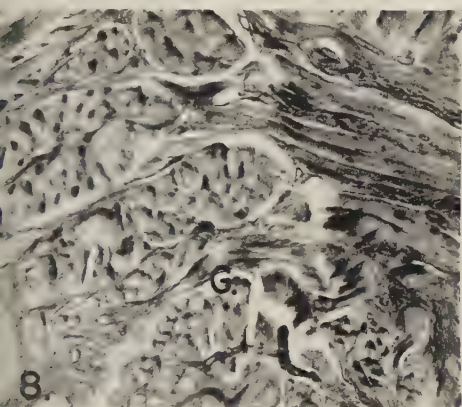
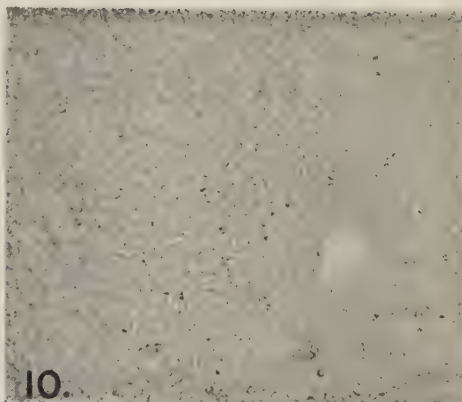
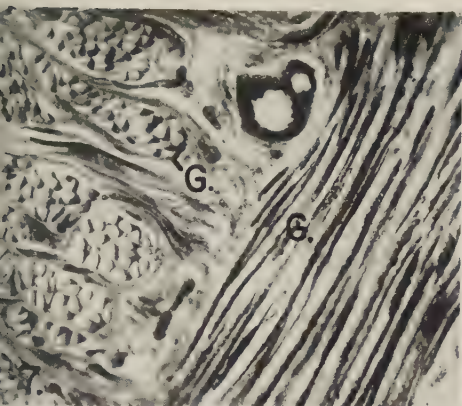


PLATE 2

EXPLANATION OF FIGURES

- 7 Striated muscle from the tongue of a warm and active Arctic ground squirrel stained by the periodic acid-Schiff method. The contained glycogen obscures the cross striations of the muscle fibers. The shift to the side of the fiber as noted in cross section is probably a fixation artifact. G = glycogen granules. (440 \times)
- 8 Striated muscle from the tongue of an animal awakened from hibernation and sacrificed stained by the periodic acid-Schiff technique. The decrease in contained glycogen allows the observation of cross striations of the fibers. G = glycogen granules. (440 \times)
- 9 Striated muscle from the tongue of an animal sacrificed in hibernation stained by the periodic acid-Schiff method. Note the negligible glycogen content and the easily apparent cross striations of the fibers. The glycogen content shown in figures 7, 8, and 9 closely parallels that shown in figures 3, 4, and 5. (440 \times)
- 10 Liver section from a typically warm and active Arctic ground squirrel stained with Sudan black B for lipids. Note the negative sudanophilia. (440 \times)
- 11 Typical liver section from an animal sacrificed during hibernation and stained with Sudan black B for lipids. Many of the parenchymal cells show the presence of fat droplets. F. d. = fat droplet. (440 \times)
- 12 Liver section from the supercooled squirrel stained with Sudan black B. Fatty degeneration is indicated by the presence of the fat globules in the parenchymal cells. F. G. = fat globule. (440 \times)



SPONTANEOUS ACTIVITY OF
THE LEMMING DICROSTONYX GROENLANDICUS
RICHARDSONI MERRIAM AS INDICATED
IN 24-HOUR RECORDS OF OXYGEN
CONSUMPTION

KENNETH C. FISHER AND MARY E. NEEDLER¹

Department of Zoology, University of Toronto, Toronto, Canada

THREE FIGURES

INTRODUCTION

Studies of the activity of animals have been made for many years. One of the earliest facts to emerge from these studies was that many types of animals were more active during one period of the day or night than another. The term "diel" has been used to designate periodic phenomena occurring in a twenty-four hour cycle (Carpenter, '38) and a large body of literature has been built up describing these phenomena. Calhoun ('44-'46) in a review of the field showed that diel cycles have been demonstrated in all the major phyla other than the Protozoa and the Porifera. It does not necessarily follow that these cycles are related to the daily cycles of light and dark, although they do appear to be so related in a number of the cases which have been investigated.

In addition to the cyclic variations with a 24 hour period, activity cycles with periods of one or only a few hours have also been found in a number of instances. Calhoun (loc. cit.) has summarized the evidence supporting the hypothesis that these short period cycles in mammals are more basic than

¹ Present address: Department of Zoology, University of California, Los Angeles 24, Calif.

those dependent on light cycles; although these latter are, temporarily at least, endogenous in the adults of many species. They are absent in the young of any species so far investigated and instead the young animals show marked short-term cycles, the diel cycles being gradually developed as the animals approach maturity. Adult animals of several species, in which the normal diel cycles have been experimentally disrupted by changing the periodicity of the light to which they were subjected, also show short term cycles. As an example of the possibility of the two types of cycles being coexistent, Calhoun's ('45) observations on *Microtus ochrogaster* may be quoted. There the feeding activity was found to have a short-term rhythmicity although the activity in an activity wheel was bimodally nocturnal.

Most of the previous work on cycles of activity in mammals has been done on forms from temperate regions. In these regions the ratio of light intensity during the day to that during the night is extremely large. In arctic regions, on the other hand, the difference between day and night for much of the year is not nearly so great. It seemed possible, therefore, that an arctic mammal might differ from a typical temperate zone species in its responses to daily variations in light and dark. It might be found for example that the diel cycles related to light, found in many rodents with more southern ranges, are not present and that as a consequence any cycle present in the shorter term variations in activity is more obvious. We undertook, therefore, an investigation relating to the activity of the arctic varying lemming.

The observations which have revealed the cyclic variations in locomotor activity under discussion here have been made in many different ways. Varshovsky ('38), for example, simply recorded the number of individuals seen above ground in an animal colony. In the investigations by Elton ('31), Hamilton ('37), Kalabuchov ('40), and others, the animals were trapped, the traps being emptied at frequent intervals.

A number of machines have been devised to measure particular types of activity under the controlled conditions

of the laboratory. They may record, as in the apparatus used by Behney ('36), the number of times which an animal passes through or over a particular place in its cage, or they may even record some particular movement of the animal. In this latter connection tilting activity cages (see par ex Richter, '27; Davis, '33; and Hunt and Schlosberg, '39) and revolving wheels (see par ex Stewart, 1898; Skinner, '33; Calhoun, '45) have frequently been employed. These methods may be adapted to record extremely small movements of the animals. None of them, however, provide measures of *total* activity. The rate of metabolism on the other hand, as indicated for example by the rate of oxygen consumption, must reflect not only gross but also small movements, as well as the differences, if any, between a sleeping and an alert, yet motionless state. The investigation reported by Pearson ('47) and Morrison ('47, '48), who worked together, is the outstanding instance in which use has been made of the rate of oxygen consumption as an intergrated measure of all activity. Observations were made continuously over a 24-hour period on each of sixteen species of small mammals. Shrews, bats, mice and flying squirrels were included in the study. The authors have shown that the amount of oxygen consumed underwent cyclic diel changes in animals considered on other grounds to show diel cycles of activity. There were as well variations which suggested the short period activity cycles found by other workers using field and mechanical methods of recording.

Therefore, in the work with the varying lemming *Dicrostonyx groenlandicus richardsoni* Merriam now to be described, information about changes of activity will be inferred from continuous measurements of the rate of oxygen consumption over 24 hour periods.

MATERIALS AND METHODS

The animals

The animals used in these experiments were varying lemmings, *Dicrostonyx groenlandicus richardsoni* Merriam,

from a colony set up in the Zoology Department of the University of Toronto in the autumn of 1952. The original stock had been live-trapped near the Defense Research Northern Laboratory at Churchill, Manitoba, in the summer of 1950 and had been maintained and bred there. The 150 animals flown south to set up the Toronto colony were all presumed to have been born in the Churchill laboratory.

The animals were maintained in galvanized iron cages 15 inches long by 9 inches wide by 7 3/4 inches high, the roof, sides, and removable floor being of 1/3 inch wire cloth. Trays below the mesh floor were also removable and were filled with sawdust to absorb urine, faeces, etc. Hay was supplied as nesting material and the cages were cleaned weekly.

The lemmings were fed daily. Throughout the period of investigation fresh carrots, cabbages, and dry oatmeal were constantly present in the cages. Milk powder had been added to the diet for a short period immediately preceding the first experimental work. Water bottles were available in a few cages but were not used by the animals, sufficient water apparently being obtained from the vegetables.

In order to avoid any variations related to oestrous cycle, all the lemmings used for this investigation were adult or large juvenile males. Most had been mated and were caged with the mates and any young which might have been born.

The room in which the colony was maintained was lighted by two windows with a southern exposure. The animals were therefore subjected to the usual daily cycles of light and dark characteristic of the Toronto latitude. The temperature of the room was kept below 15°C. by an automatic refrigeration unit. It varied between 9° and 15° C., temperatures being generally lower at night.

The respirometer

At the beginning of this study a survey of the literature indicated that three arrangements had been described which appeared particularly useful for the continuous measurement of the oxygen consumption of small mammals over twenty-

four hour periods, (Werthessen, '37; Lewis and Luck, '33; Morrison, '47). It seemed possible, however, that inherently simpler and more robust arrangements could be devised. The device actually employed for the present experiments therefore differs somewhat from any previously described. With it, oxygen utilization is measured by counting drops of saline which are drawn into the reservoir from which the animal is using oxygen.

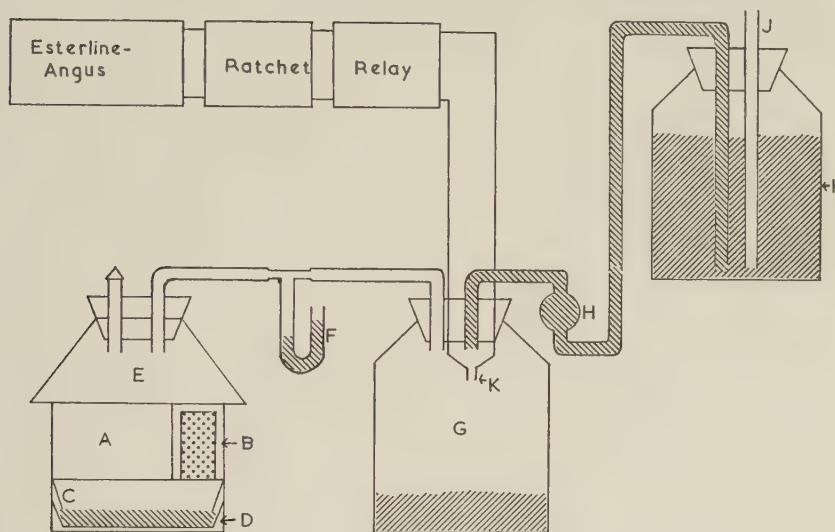


Fig. 1 Diagram of the continually recording respirometer used

- | | | |
|-----------------------|---------------------------|----------------------------|
| A, animal chamber | E, air-filled desiccator | I, salt solution reservoir |
| B, Baralyme | F, water-filled manometer | J, outlet to atmosphere |
| C, tinsheeting funnel | G, oxygen reservoir | K, platinum wires |
| D, paraffin oil | H, glass bulb of siphon | |

A schematic diagram of the new respirometer is given in figure 1. The animal was enclosed in a galvanized wire cage (A) of one-quarter inch mesh within a Pyrex desiccator (E). The circular cage had a diameter of approximately 9 1/2 inches and a height of 3 1/2, the largest possible within the ten inch diameter of the desiccator. A tin sheeting funnel (C) of the diameter of the cage was welded to its bottom and stood in a tray of paraffin oil (D). Faeces and other debris dropped

through the mesh floor into the oil, preventing fumes from forming and the consumption of oxygen by putrefaction of the organic matter. A cage of this type provided sufficient space so that enough food could be left in the cage to supply the animal for the duration of the experiment, and permitted the animal to move freely.

A galvanized wire mesh wall cut off a small section of the cage, within which a 1/16 inch copper mesh inner basket (B) contained carbon dioxide absorbing pellets. A lid on the larger section prevented the animal from reaching the pellets. These pellets were of a commercial preparation, Baralyme (Thomas Edison Co.), which is an indicating mixture of barium octohydrate and calcium hydroxide. That it was completely efficient was shown by analyzing samples of the chamber gas with Fry's ('49) modification of Krogh's microgas analyzer. No carbon dioxide could be detected by this method, when samples were taken at random during the course of several experiments.

The vacuum created by the oxygen consumption caused oxygen to be drawn over from a connecting reservoir (G) of pure oxygen, so that the normal oxygen : nitrogen ratio was maintained in the animal chamber. This in turn tended to produce a vacuum in the oxygen reservoir which was, in its turn, filled by saturated sodium chloride solution drawn across a siphon from a second reservoir (1). Finally, a tube (J) to the outside allowed the second reservoir to fill with air as the salt solution was removed.

In order to facilitate the starting of the siphon at the beginning of the experiment it was found necessary to have a glass bulb (H) inserted in the siphon and to have a slightly higher gas pressure operating throughout the system than the atmospheric pressure outside it. The extent of this extra pressure (always less than 0.05 cm Hg) was watched by inserting a small water-filled manometer (F) between the animal cage and the oxygen reservoir.

As each drop of saline entered the oxygen reservoir it passed between two platinum wires (K) thus completing a

circuit between them. This contact was in the grid circuit of a vacuum tube which was so connected as to pass plate current while the contact was made. The plate current of the vacuum tube was used to operate a pen on a moving-paper recorder. (Esterline-Angus Co. operation recorder). Thus the number of drops entering the reservoir in any desired interval of time could be counted. In order to reduce the labour involved in actually counting each drop, it was found convenient to record only each twentieth drop. This was done by arranging the individual drops to operate a "stepper." In this arrangement each drop, by means of a ratchet, caused a shaft to rotate one-twentieth of a revolution. A contact on this shaft operated once per revolution and was used to operate the recorder pen which then signalled each twentieth drop.

In such an animal chamber the humidity became 100% very rapidly after the animal had been put in, and condensation of water on the glass walls occurred. However, relatively little water condensed on the metal cage in which the animal was enclosed, and the animals were not wet when taken from cage at the end of an experiment. The condensation prevented direct observation of the animal during an experiment.

The animal chamber and the oxygen reservoir were contained in a wooden cabinet in which the temperature was regulated to within 1°C. The temperature within the desiccator varied between 1° and 2°C. above that of the cabinet. Double glass windows on the front of this chamber allowed normal daily light cycles within it. Before even momentarily turning on room lights after dark, a blind was drawn across these windows to prevent disturbance of the experiment.

Since the reservoir of salt solution which is a part of this respirometer was open to the air, the pressure on it varied with the barometric pressure. Hence, the differential pressure between it and the animal chamber was not influenced solely by oxygen utilization. A recording barometer was therefore operated continuously so that corrections for barometric pressure changes could be made if desired. In fact, however, the correc-

tions indicated in the experiments to be described were completely negligible.

In order to convert the data obtained from the number of drops of saline pulled across the siphon, into the volume of oxygen consumed, the volume of oxygen per drop was calculated at each experimental temperature. The average drop weight was found from the weight of 200 drops for each temperature, and this was divided by the specific gravity of the salt solution. These conversion factors were .0712 cm³ oxygen per drop at 20°C., .0698 cm³ at 25°C., and .0685 cm³ at 30°C.

Experimental procedure

The oxygen reservoir was placed in the temperature controlled cabinet and was filled with oxygen by twice evacuating it with a vacuum pump and refilling from a cylinder of pure oxygen. The experimental animal was weighed and placed in the animal cage in the desiccator together with enough food and water to supply it for twenty-four hours. The desiccator was then placed in the temperature controlled cabinet. The heat given off by the animal warmed the gas in the desiccator thus causing it to expand. As soon as it was thought that sufficient equilibration had taken place so that the expanding gases would not later back up the siphon — a period of 1 to 3 hours — the latter was connected to the desiccator and oxygen reservoir.

The durations of the individual tests were at least twenty-four hours and in some experiments 48 hours. Data were obtained at 20°C. and 25°C. and a few experiments were run at 30°C. although this temperature brought about the death of some individuals.

RESULTS

In figure 2 the average rate of oxygen consumption during each 20 minute interval of a period of 24 hours is shown. It will be noted that the first two measurements (A and B) were lower than any others. This happened frequently and was presumed

to indicate that equilibration had not been quite complete, each experiment having been started as soon as it was possible to maintain the siphon running.

In determining the end of equilibration it was assumed that the first point (C) on the graphs which was higher than at least one point later on the graph was unaffected by this "equilibration lag." Each twenty-four hour period in the results to be discussed was begun at this point. For example, the twenty-four hour period which was used for analysis in the experiment represented in figure 2 extended from C to D.

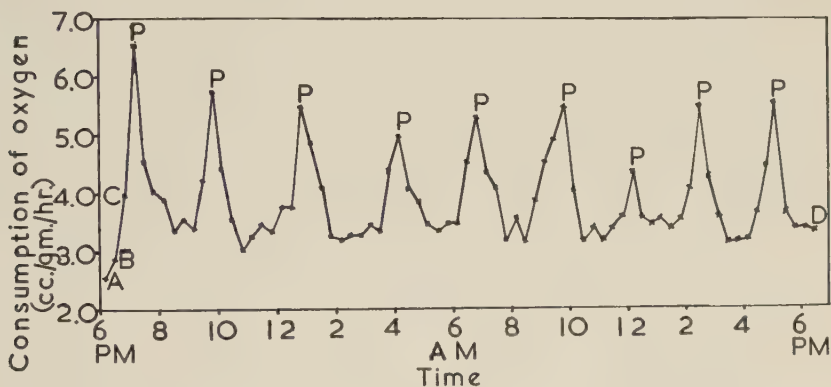


Fig. 2 The rate of consumption of oxygen in a typical experiment. Each value taken as a "peak" is indicated by the letter P. Points A, B, C, and D are referred to in the text.

As the initial step in the examination of the observations, the total oxygen consumed in the first half of each experiment was compared with that consumed in the second half. This comparison would presumably reveal any further equilibration which took place. It would also be possible in this way to detect any tendency for the animal to undergo any form of adaptation to the enclosure which might lead to a change in the absolute amount of activity as reflected by metabolism. So that a breakdown in this way into halves would not represent a breakdown into night and day observations, experiments were started at times evenly distributed over the interval from

noon to 6 A.M. On the average the consumption of oxygen in the second half was 3% greater than in the first half. Using the "t" test for paired variates it was found that the probability of a chance occurrence of this difference between the two halves of the experiments was 0.2 ($N=16$).

The actual average daily rates of oxygen consumption for nine different animals under various temperatures are shown in table 1. The data are insufficient to establish the point beyond question, but it seems evident (as suggested in the

TABLE 1
The rate of oxygen consumption by lemming
($\text{cm}^3/\text{gm}/\text{hr}$)

| ANIMAL NO. | TEMPERATURE | | | WEIGHT ¹ |
|------------|-------------|-------|-------|---------------------|
| | 20°C. | 25°C. | 30°C. | |
| 89 | 3.29 | 3.66 | 3.66 | 80 |
| 215 | 4.56 | 4.07 | 3.87 | 55 |
| 87 | 4.37 | 4.49 | .. | 83.5 |
| 220 | 3.88 | 5.78 | .. | 67.5 |
| 189 | 3.20 | 3.50 | .. | 83 |
| 101 | 3.89 | .. | .. | 67 |
| 44 | .. | 3.75 | .. | 80 |
| 84 | .. | .. | 3.06 | 99.5 |
| 183 | 4.39 | .. | .. | 67.5 |

¹Lowest weight found for the animal in any experiment.

work of Scholander, Walters, Hock and Irving, '50, on the subspecies *D. g. rubricatus*) that the rate of oxygen consumption by the lemming is reasonably constant from 20°C. to 30°C. The average rate of 4.08 c.c. per gram per hour indicated for the lemming by these data is very similar to the values recorded by Pearson ('47) for a number of other small mammals.

Inspection of the individual graphs of which figure 2 is an example does not suggest the presence of any diel cycle. However, to obtain quantitative evidence on this question, the total oxygen consumed during the night (i.e., from 6 P.M. to 6 A.M.) in each experiment was compared with that consumed during the day (i.e., 6 A.M. to 6 P.M.). The ratios of these varied from

0.9 to 1.1 and averaged 1.01. A "t" test showed that there was no difference between the amounts of oxygen consumed during day and night ($P =$ approximately 0.4, $N = 16$). There is then no suggestion whatever that on the average the lemming has a diel cycle. It is to be noted that the animals used in this investigation had been subjected from birth to the daily cycles of light and dark normal to the Toronto region. The implication is that there is not even a latent ability for the establishment of a diel cycle.

While there is no evidence for diel cycles of activity in the lemming from these measurements of oxygen consumption, there are very obvious indications of remarkable short term cycles. Peaks in the rate of oxygen consumption in data such as those of figure 2 occurred as close together as one hour in some instances and were as far apart as $4\frac{2}{3}$ hours in others. The peaks represent on the average an increase of about 50% in the rate of oxygen consumption above the value in the troughs, the latter presumably representing the resting rates.

In order to examine the properties of this short term cycle, the position of each peak on each 24 hour graph was determined by inspection and was marked as in figure 2. The time from the first peak to the last divided by the number of cycles included in this interval was taken as the average cycle duration in the experiment in question. These durations varied from one experiment to another from 2.22 to 3.86 hours.

To permit comparisons of the cyclic variations in the different experiments, the bar-graph of figure 3 was constructed. The time at which each peak occurred in any one experiment is shown by a vertical line, considering the first peak of that experiment as occurring at zero time. The experiments were arranged in order, those having the shortest average cycles at the top and so on. To emphasize the constancy from one experiment to another of the cyclic variation seen in each experiment, the space between each alternate peak and the following one was cross hatched. It will be apparent from the diagram that there was a basic regular pattern.

Four of the animals reported on in figure 3, namely numbers 87, 89, 215 and 220, were each used more than once for respiratory experiments. It is therefore possible to obtain from them an estimate of the variation of the cycle duration from experiment to experiment in an individual. For these four the differences between the longest and the shortest average cycle duration may be seen to be 0.22, 0.06, 0.64, and 0.17 hours respectively. It is indicated that the average cycle

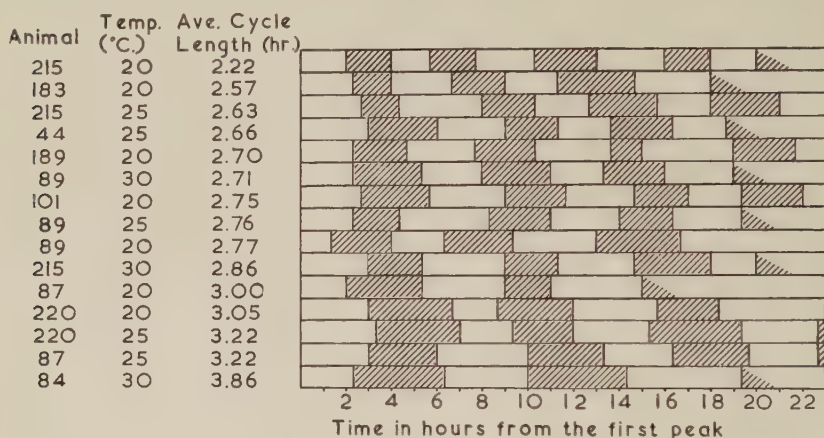


Fig. 3 The interval between peaks. The time at which each peak occurred in any one experiment is shown by a vertical line, considering the first peak of that experiment as occurring at zero time. The experiments are arranged in order, those having the shortest average cycle being at the top. The interval between the second and third, fourth and fifth, sixth and seventh, etc., peaks has been cross-hatched to emphasize the cycle lengths.

duration tends to be less variable within an individual than between individuals because, as noted above, the variation of the average cycle duration considering all of the experiments in figure 3 is from 2.22 to 3.86 or 1.64 hours.

There are no indications in figure 3 that the cycle duration varies over the temperature range 20° to 30°C. However, the data may not be numerous enough to detect a small variation.

Pearson ('47) found short term metabolic cycles in various shrews and mice, but none showed such a consistently regular periodicity as is here seen in lemming. *Microtus*, the meadow

vole, provided the record most similar to that for lemming, and it, of all the animals on which he was working, is also the most closely related to *Dicrostonyx*.

Various investigators have attempted to relate the short term activity rhythms to other physiological characteristics of the organism. Thus Pearson (loc. cit.) compared the metabolic cycles he observed in *Microtus* to the short term cycles in food-getting activity found for this animal by Hatfield ('40), and Richter ('27) postulated that the large stomach contractions found at the beginning of each short term cycle in the rat actually initiated the cycle of activity. However, Munn ('50) reviewed work on rats which tends to show that periodic activity occurs after removal of the stomach and also after cutting the nerves from the nerve centers. Furthermore, he notes that Richter's rats fed at the *end* of each short term cycle of activity. Crowcroft ('54) working on three British species of shrews found that, although the shrews exhibited rather irregular short term rhythms of the order of 2 hours in general activity, the most frequent periods of feeding activity were much shorter than this. Although the shrews fed soon after waking, other activities usually preceded feeding. It may be suspected from all this that the animal's activity is not necessarily directly related to a desire to feed.

Other investigators have suggested a relation between the length of the short term cycle and the general metabolic rate of the animal (see for example Calhoun, '44). In similar vein Crowcroft ('54) in an undocumented summary states that the period of short term rhythms in rodents decreases as the size of the species decreases. He gives 4 hours as the length of the cycle in *R. norvegicus*, 2 1/2 hours in *Microtus*, 2 hours in *Apodemus* and *Peromyscus*, and 3/4 to 1 1/2 hours in *Mus musculus*. It must be noted, however, that in Morrison's ('48) list of the lengths of short term cycles, there does not appear to be any correspondence between weight and cycle length or between average rate of oxygen consumption (i.e., metabolic rate) and cycle length.

As suggested by Calhoun ('45-'46), it is possibly more useful to think of the short term cycle of locomotor activity as a very basic innate character. It might be supposed that through the processes of evolution this cycle had been modified so as to provide that periods of locomotion would occur at least as frequently as was required for any other activity of the animal which depended on locomotion. Thus a rough correspondence between activity cycles and feeding cycles might exist but not a close approximation of the two; and similarly, some suggestion might be found of a relationship between general metabolic rate, with which feeding is connected, and the length of the cycle. Some such view as this seems required to resolve the inconsistencies apparent in some of the interpretations referred to above.

SUMMARY AND CONCLUSIONS

1. A new type of respirometer has been designed, by which the oxygen consumption of small mammals may be observed over prolonged periods. It records electrically the number of drops of salt solution drawn across a siphon from a reservoir by the reduced pressure within the respiration chamber which is caused by the consumption of oxygen by the animal, when carbon dioxide is absorbed.

2. The rate of oxygen consumption by the varying lemming *Dicrostonyx groenlandicus richardsoni* Merriam has been measured continuously over periods of 24 hours at 20° and 25°C. A few observations were made at 30°C. as well.

3. The rate of metabolism seemed to be independent of temperature from 20° to 30°C., the average rate of oxygen consumption being 4.08 cm³/gm/hr.

4. No diel rhythm in the rate of oxygen consumption was found, and this presumably indicates that there is no diel rhythm of locomotor activity in this animal.

5. Very marked short term cyclic variations in the rate of oxygen consumption were found, the pattern of the variations being very regular. The average cycle duration in 24 hour experiments varied from 2.22 hours to 3.86 hours. Presumably

this indicates marked cyclic variations of locomotor activity in the lemming.

ACKNOWLEDGMENTS

Thanks are extended to the Defense Research Board of Canada, and especially to the officers of its Northern Laboratory, who arranged for the supply of varying lemmings from which the colony at Toronto was begun. The experimental work was carried forward with financial assistance from a grant by the Research Council of Ontario to Professor J. R. Dymond and a grant from the National Research Council of Canada to Dr. K. C. Fisher. Miss M. E. Needler held a bursary from the National Research Council of Canada and a scholarship from the Research Council of Ontario during this investigation.

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Note added in proof: R. Hansen has recently published data which show a short term activity cycle in the varying lemming (*Jour. Mammal.* 38: 218-223, 1957).

CHANGES IN CELL VOLUME IN RELATION TO AGE OF CULTURES AND OTHER FACTORS IN CERTAIN PROTOZOA AND BACTERIA¹

JOHN J. CORBETT²

Biological Laboratories, New York University

TEN FIGURES

Protozoa and bacteria have been shown to undergo similar growth phases in cultures (Hall, '53; Wilson and Miles, '46), and comparable variations in average cell volumes and enzyme production have been reported for different periods in the growth of populations (Ormsbee, '42; Wilson and Miles, '46). It was felt that a consideration of such variables might produce significant data bearing on problems of adaptation. As a basic procedure, it was decided to trace changes in volume which might occur when Protozoa and bacteria were transferred to various fresh media from stock cultures in different phases of growth.

MATERIALS AND METHODS

The organisms employed were *Euglena gracilis* (Damon strain, *Astasia longa*, *Chilomonas paramecium* (Pringsheim strain), *Tetrahymena pyriformis* (strain H), *Escherichia coli communis*, *E. coli mutabilis* (strain ML-3b), *Streptococcus faecalis* (strain ATCC 8043), *Lactobacillus fermentum* (strain ATCC 9833), and *L. leichmannii* (strain ATCC 7830).

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²Present address: Department of Biology, Manhattan College, Riverdale, New York City.

The following media were used for Protozoa:

Medium A (Hutchens, Jandorf, and Hastings, '41): NH_4Cl , 450 mgs; Na-acetate, 2500 mgs; $(\text{NH}_4)_2\text{SO}_4$, 100 mgs; K_2HPO_4 , 1950 mgs; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 20 mgs; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 15 mgs; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 3 mgs; thiamine hydrochloride, 10 mgs in 1 liter H_2O (w/v); pH 6.8.

Medium B: Peptone, 1 gm; Na-acetate, 2 gms; thiamine hydrochloride, 10 mg; cyanocobalamin, $1\text{ }\mu\text{g}$ per liter; pH 6.8.

Medium C: Synthetic medium for *Tetrahymena* (Kidder and Dewey, '47) with 1 unit of protogen per liter. In various experiments, these media were modified as specifically indicated.

For the various bacteria, basal media (Difco) for assays of appropriate vitamins were employed with addition of the required vitamin. Thus, folic acid, thiamine, and vitamin B_{12} were added for *S. faecalis*, *L. fermentum*, and *L. leichmannii*, respectively. In addition, the synthetic medium of Monod and Wollman ('47) was used for *E. coli*. This medium was supplemented with dextrose or lactose, as required in particular experiments.

Counts of Protozoa were obtained with a Sedgwick-Rafter counting chamber. Bacterial growth was measured either as optical density with a Lumetron, or as total volume of cells per ml. Cell volumes were measured by means of hematocrit tubes and expressed as total cellular volume per ml or as average volume per cell calculated from the number of cells per ml. In certain experiments, as noted below, the cell volumes of Protozoa were calculated from microscopic measurements of length and width.

Densities of flagellates were determined for *E. gracilis* and *A. longa* by weighing the centrifuged cells in a tared 15 ml tube. Previous to weighing, the walls of the tube were dried with filter paper and the cells were dried by exposure to a current of air at very low velocity for 2 minutes. After weighing, the cells were immediately resuspended in a measured volume of medium, shaken, and counted. Weights of

dry residue per cell were obtained by heating cellular masses at 85°C. over aluminum oxide for 18 hours.

Bacterial cultures were incubated at 37°C., and suspensions were stored at 9°C.; *E. gracilis* and *A. longa* were incubated at 26—27°C., *C. paramecium* at 30—31°C., and *T. pyriformis* at 27—28°C. Protozoan cultures also were stored at 9°C.

EXPERIMENTAL RESULTS

Changes in cell volumes following transfer to fresh homologous media

Preliminary observations on suspensions of resting organisms revealed a diminution of cellular volumes in fresh media. In two cases, the total volume (mm³/ml) had decreased after 2—3 hours from 3.12 to 2.08 for *L. leichmannii* and from 3.25 to 1.27 for *S. faecalis*. In another case, suspensions of *L. leichmannii* showed a similar decrease from 3.81 to 3.09, and then an increase to 3.41 after 28 hours.

TABLE 1

| GROWTH PHASE | % VOLUME CHANGE | |
|-------------------|-----------------|---------|
| | 1.5 hrs. | 24 hrs. |
| Early logarithmic | — 17.5 | — 10.0 |
| Logarithmic | — 11.0 | 0.0 |
| Maximal density | + 39.0 | + 43.0 |
| Decline | — 2.5 | + 21.0 |

L. fermentum from cultures in different growth phases was resuspended and relative volumes were determined at intervals for 21 hours (fig. 1). Wherever initial contraction was observed, there was a subsequent return to approximately initial volume. Also, initial contraction was maximum in cells from the early logarithmic phase.

Similar determinations were made on *E. coli mutabilis* in synthetic medium with 0.2% dextrose (table 1). It is noteworthy that bacteria from cultures which had attained maximum density underwent an initial expansion. Thus it is evident that the change in volume of bacteria upon transfer

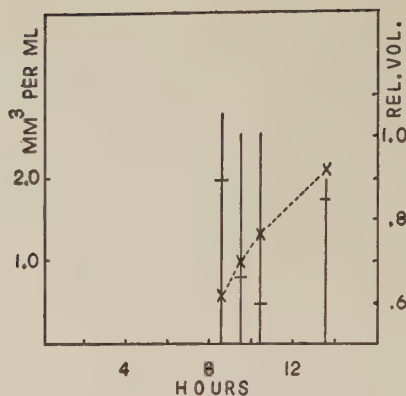


Fig. 1 Changes in volume of *Lactobacillus fermentum* after transfer from a stock culture in different stages of growth to fresh homologous medium at 9°C. The broken line, joining points indicated by "X", traces growth of the culture expressed as total volume of cells per milliliter. Each vertical line, with the short horizontal line transecting it, records the changes in relative volume observed in suspensions made at the point indicated. The position of the horizontal bar marks the degree of contraction; height of the vertical line, the extent of subsequent increase in relative volume.

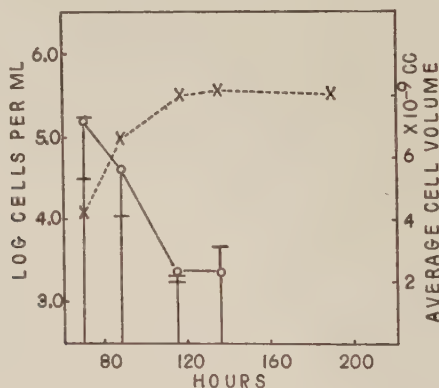


Fig. 2 Changes in average volume of *Euglena gracilis* during growth of a culture and after transfer of suspensions to fresh medium B at 9°C. The solid line joining the circles traces the progressive decrease in average volume during growth. The broken line traces growth of the population (log cells/ml). The vertical lines joining the circles indicate the points at which suspensions of the flagellates were transferred to fresh medium. At the first three points, a horizontal bar marks the extent of contraction; in the first and third cases, a second bar indicates the volume at equilibrium after reexpansion to approximately the initial average volume. Flagellates suspended after 137 hours of incubation showed an initial expansion instead of a contraction.

to fresh media varies according to the growth phase of the parent culture.

In figure 2, progressive changes in average cell volume of *E. gracilis* during growth and the changes occurring after transfer of suspensions to fresh homologous medium are correlated with growth of the population in a single culture in medium B. In table 2, maximal changes in volume are recorded for similar suspensions of *E. gracilis* from cultures of different ages and population densities.

TABLE 2

Changes in average volume (as percent of average initial volume) of Euglena gracilis from cultures of different ages and population density after suspension in fresh medium B at 9°C.

| AGE OF CULTURE | CELLS/ML | MAXIMUM VOLUME CHANGE |
|----------------|----------|-----------------------|
| <i>hrs.</i> | | % |
| 65 | 12,720 | — 6 |
| 68 | 17,230 | — 44 |
| 70 | 33,100 | — 21 |
| 89 | 87,300 | — 11 |
| 115 | 116,800 | — 20 |
| 135 | 284,500 | — 16 |
| .. | 302,000 | — 13 |
| .. | 385,000 | + 10 |
| 140 | 388,000 | + 32 |

Comparable results were obtained with *Chilomonas paramecium* from the early logarithmic phase in medium B and resuspended in the homologous medium. The average initial volume was $1.23 (\pm 0.09) \times 10^{-6} \text{ mm}^3$. After one hour at 9°C., the volume of flagellates suspended in fresh medium dropped to $0.77 (\pm 0.04) \times 10^{-6}$; after 20 hours at the same temperature, the volume in similar suspensions showed an increase to $1.45 (\pm 0.15) \times 10^{-6} \text{ mm}^3$. These data provide evidence that, just as in bacteria, Protozoa transferred to fresh medium undergo either contraction and reexpansion or an initial expansion, depending upon the growth phase of the parent culture.

*Changes in volume after transfer to fresh
heterologous media*

Tetrahymena pyriformis in medium C was transferred from cultures at maximum density to the same medium and to a modified medium in which L-arginine was replaced by twice its chemical equivalent of DL-ornithine. The expected initial expansion occurred in the homologous medium (fig. 3).

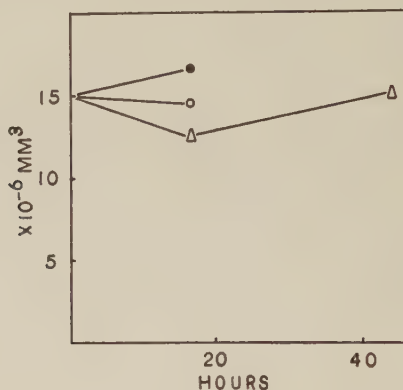


Fig. 3 Changes in volume of *Tetrahymena pyriformis* H from a culture at maximal density in medium C. The closed circle marks the change in volume after suspension and storage at 9°C. for 18 hours in fresh homologous medium; the open circle, volume after suspension and storage in the supernatant culture fluid as a control. The triangles indicate the volumes in modified medium (ornithine replacing arginine) after storage for 18 and 42 hours.

When arginine was replaced by ornithine, the ciliates showed a marked initial contraction; after 42 hours, the ciliates had reexpanded to approximately their initial volume.

A similar experiment was performed with *Euglena gracilis* at the logarithmic phase in medium B. Suspensions in the homologous medium and in medium A were stored at 9°C. The expected small degree of contraction occurred in the homologous medium, in contrast to a pronounced contraction of short duration in the synthetic medium (table 3).

Escherichia coli communis, from the maximum density phase in synthetic medium plus 0.2% dextrose, was resuspen-

TABLE 3

Changes in volume of *Euglena gracilis* from a logarithmic phase culture in medium B. Suspensions in supernatant culture fluid (control), in fresh medium B, and in medium A were stored at 9°C.

| TIME hrs. | RELATIVE CELL VOLUMES | | |
|--------------|-----------------------|----------|----------|
| | Control | Medium B | Medium A |
| 0.25 | 1.00 | 0.92 | 0.39 |
| 1.0 | 1.03 | 1.15 | 0.40 |
| 3.0 | 0.89 | 1.43 | 1.27 |

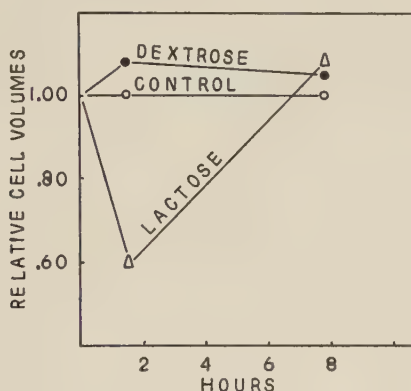


Fig. 4 Changes in volume of *Escherichia coli communis* from a culture at maximal density in synthetic medium plus 0.2% dextrose. Suspensions were stored at 9°C. in the supernatant culture fluid (control), in fresh homologous medium (dextrose), and in synthetic medium plus 0.2% lactose.

ded in fresh homologous medium, in synthetic medium plus 0.2% lactose, and also in the supernatant culture fluid as a control. The expected initial expansion occurred with dextrose (fig. 4); with lactose, there was an initial contraction and then a subsequent expansion of the unadapted organisms.

Factors influencing reexpansion or initial expansion

Possible effects of α -methylglucoside on expansion were tested with *E. coli communis* from a culture at maximal density in synthetic medium plus dextrose. In the presence of

α -methylglucoside, which is an inhibitor of enzyme production, a marked decrease in volume occurred with dextrose as well as with lactose and reexpansion of the bacilli had not occurred at the end of 28 hours (fig. 5). These data seem to suggest a dependence of expansion upon enzyme activity.

E. coli mutabilis ML-3b (lactose-negative) was grown in the synthetic medium with dextrose and, at different growth phases, the cells were resuspended in the homologous medium and in synthetic medium with 0.2% lactose. As indicated in

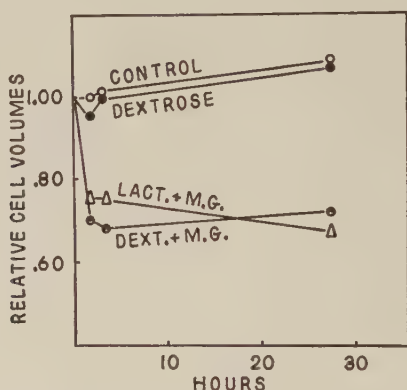


Fig 5 Changes in volume of *Escherichia coli communis* from a culture at maximal density in synthetic medium with 0.2% dextrose. Suspensions were stored at 9°C. in supernatant culture fluid (control), in fresh homologous medium (dextrose), and in synthetic media containing α -methylglucoside and lactose and dextrose.

figure 6, it was evident that, in every case, the cell volume at equilibrium in lactose medium was considerably less than that of cells transferred simultaneously to dextrose medium.

In order to determine the possible inhibition of β -galactosidase formation by low temperature (9°C.) *per se*, a colony of the lactose-positive variant of *E. coli mutabilis* ML-3b from Endo's agar was subcultured in lactose-synthetic medium. After 15 hours of incubation, the bacilli were resuspended in fresh homologous and dextrose medium and stored at 9°C. After 2.5 hours, an initial expansion was already evident

in the lactose medium (table 4), suggesting that this variant was producing β -galactosidase and also that failure of the lactose-negative variant to produce the enzyme was not a result of the low temperature *per se*. The observed behavior of this strain was to be expected in the light of claims that lactose adaptation involves a selection and multiplication of spontaneous mutants.

The effects of dextrose and lactose on the contraction and expansion of *E. coli communis* were also compared on bacilli which were sugar-depleted. A culture in synthetic medium with dextrose (0.004M), which reached maximum density at

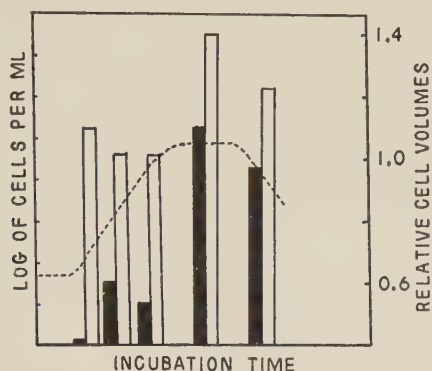


Fig. 6 Changes in volume of *Escherichia coli mutabilis* (lactose-negative) in transfers from different growth phases in dextrose-synthetic medium to the homologous medium (open bar) and to lactose-synthetic medium (solid bar). Height of bars indicates final equilibrium volume, and dotted line indicates respective growth phases.

TABLE 4

Changes in relative volume of a lactose-positive variant of *Escherichia coli mutabilis* ML-3b, grown in synthetic medium plus lactose. Suspensions were stored at 9°C. in the supernatant culture fluid (control) and in synthetic media supplemented with lactose or dextrose.

| SUSPENSION | RELATIVE VOLUMES OF CELLS/ML | |
|------------|------------------------------|----------|
| | 2.5 hrs. | 5.0 hrs. |
| Control | 1.00 | 1.00 |
| Dextrose | 0.92 | 1.35 |
| Lactose | 1.08 | 1.24 |

a relatively low level, served as the source of organisms for transfers to the same medium and to synthetic medium with 0.004M lactose. As indicated in figure 7, immediate expansion of the cells occurred in lactose medium, whereas there was a prolonged initial contraction with a chemically equivalent concentration of dextrose. The results might suggest that expansion of the depleted cells depended on the number

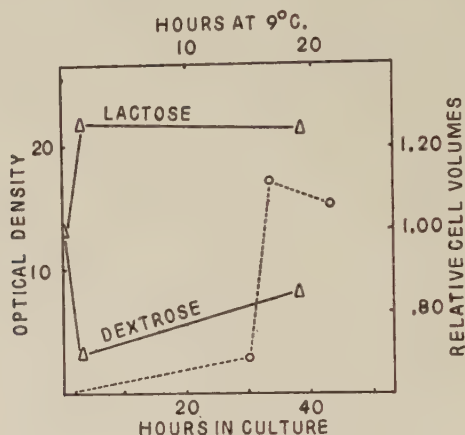


Fig 7 Changes in volume of *Escherichia coli communis* from a culture in synthetic medium with dextrose at a suboptimal concentration (0.004M). The broken lines trace growth of the stock culture. At maximal density, transfers were made to the homologous medium (dextrose) and to synthetic medium with lactose (0.004M).

of particles produced per mole of the sugar, a single molecule of disaccharide yielding two molecules of monosaccharide. In other words, expansion may be related to the number of particles entering the cell.

Euglena gracilis was chosen as the test organism for determining the possible influence of temperature on the rate of reexpansion following an initial contraction. Flagellates were harvested from cultures in medium B at early logarithmic phase, resuspended in the homologous medium, and then stored at different temperatures. On the basis of microscopic measurements of length and width, volumes were calculated

from $\frac{L+W}{2}$ and the data were tested for statistical significance. It is evident that, with higher temperatures, reexpansion was completed within shorter times (fig. 8). It is noteworthy, also, that the slope of the curve diminishes above 26°C., which is optimum for growth of the species.

Euglena gracilis also was employed to study the relation of substrate concentration to the time required for reexpansion. Flagellates were harvested from cultures in medium A and medium B at logarithmic phase and transferred to normal and modified homologous media. Suspensions were stored

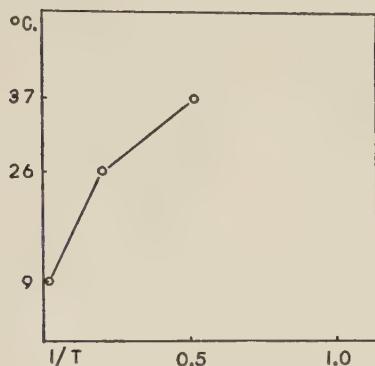


Fig. 8 Changes in volume of *Euglena gracilis* in relation to temperatures at which suspensions were stored. Temperature is plotted against the reciprocal of the time required for reexpansion to the original volume after initial contraction.

at 9°C. in half-strength and normal medium A and in normal, double- and triple strength medium B. After storage for 2-3 hours, greater relative volumes were observed with higher concentrations of substrates in each type of medium.

Euglena gracilis was used to determine the possible influence on reexpansion, or initial expansion, of general conditions within the medium which might limit the action of enzymes and availability of substrates. *E. gracilis* from a culture at maximum density in medium B was resuspended in the supernatant culture fluid after addition of sodium acetate at levels of 0.01 and 0.02 M. In these suspensions, the flagellates underwent a

contraction, which was greater with the higher concentration of acetate. It was assumed that the failure to expand in response to renewal of a single substrate might be due to the physiologically unfavorable condition of the culture medium in the maximum-density phase (pH, oxidation-reduction potential, etc.).

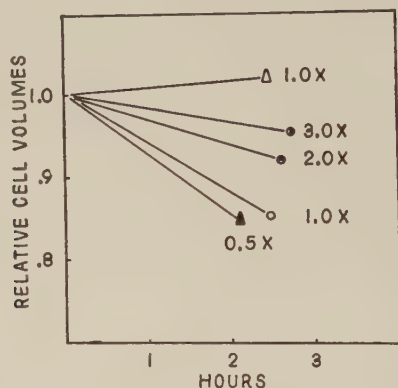


Fig. 9 Influence of substrate concentration on rate of reexpansion in *Euglena gracilis*. Suspensions from a logarithmic phase culture in medium A were stored in half-strength (0.5X) and in normal (1.0X) medium A (triangles); suspensions from a culture in medium B were suspended in medium B with the components at 1.0, 2.0, and 3.0 times the normal concentrations.

A further test was applied to determine whether failure of expansion might result from the absence of a required vitamin, which would normally be present in only trace concentrations. *E. gracilis* was grown in medium A supplemented with 0.01 mμg vitamin B₁₂ per ml, and this culture, which attained a low maximum density, was used as a source of vitamin-depleted flagellates. Subsequent suspension of the flagellates in medium containing excess B₁₂ (15 mμg per ml) gave rise to expansion at 9°C., whereas those in B₁₂-free medium underwent a pronounced initial contraction and failed to attain a volume comparable to that of the controls at any time during the experiment (table 5).

In a further experiment with *E. gracilis*, an attempt was made to correlate changes in cellular density with changes

TABLE 5

Influence of vitamin B₁₂-depletion on volume changes in Euglena gracilis

| TIME AT 9°C. | RELATIVE CELL VOLUMES | |
|--------------|-----------------------|-----------------------|
| | + B ₁₂ | B ₁₂ -Free |
| <i>hrs.</i> | | |
| 0 | 1.00 | 1.00 |
| 2 | 1.08 | 0.57 |
| 5 | 1.03 | 0.60 |
| 24 | 1.34 | 0.75 |
| 30 | 1.27 | 0.93 |
| 96 | 1.19 | 0.64 |

TABLE 6

Changes in average cell volume, weight and density of Euglena gracilis after transfer to fresh homologous media. Except where indicated otherwise, suspensions were stored at 9°C.

| MEDIUM | HRS. IN SUSPENSION | AVERAGE CELL VOLUME (mm ³ × 10 ⁻⁶) | WT./CELL (mμg) | DENSITY |
|----------|-----------------------|---|-------------------|---------|
| A | 0 | 4.46 | 6.12 | 1.37 |
| | 2.5 | 7.70 | 7.49 | 0.97 |
| B | 0 | 5.96 | 5.38 | 0.90 |
| | 1.5 | 3.66 | 5.20 | 1.42 |
| | 18 | 4.90 | 5.48 | 1.12 |
| B | 0 | 5.65 | 5.99 | 1.06 |
| | 1.5 | 4.10 | 5.19 | 1.27 |
| | 24 | 4.85 | 4.58 | 0.94 |
| B (0°C.) | 0 | 2.36 | 3.37 | 1.42 |
| | 1.5 | 2.60 | 2.70 | 1.03 |

in volume. Table 6 summarizes the data obtained with two different cultures, one in medium A and the other in medium B. Suspensions in fresh homologous medium were made from the first culture at the phase of maximal density; from the culture in medium B, at the logarithmic phase. Although the data are insufficient for very accurate determinations, it is obvious that contraction is consistently accompanied by relatively high density of the cells, while reexpansion (or initial expansion) is accompanied by a reduction in density. Such findings

would be expected if the cells lose water during contraction and gain water during expansion.

In order to obtain information concerning relative amounts of intracellular water in early logarithmic cultures and in those which had attained maximal density, determinations of density were made on appropriate cultures of *Euglena gracilis*, *Astasia longa*, *E. coli communis*, *E. coli mutabilis* ML-3b, *Staphylococcus aureus*, and *Lactobacillus casei* (table 7). An increased density in older cultures is evident for all of these organisms. The exceptionally low density for early logarithmic *A. longa* might be due to loss of flagellates in removal of

TABLE 7
Changes in density of bacteria and Protozoa between the early logarithmic and maximal density phases of growth

| ORGANISM | DENSITY | |
|-------------------------------|-------------------|-----------------|
| | Early logarithmic | Maximal Density |
| <i>Euglena gracilis</i> | 1.054 | 1.420 |
| <i>Astasia longa</i> | 0.810 | 1.247 |
| <i>E. coli communis</i> | 1.143 | 1.325 |
| <i>E. coli mutabilis</i> ML3b | 0.903 | 1.370 |
| <i>Staphylococcus aureus</i> | 1.042 | 1.270 |
| <i>Lactobacillus casei</i> | 1.068 | 1.274 |

the supernatant culture fluid, which is more likely to occur with those of a density approximating that of the suspending medium. Also, determinations of loss in weight on drying at 85°C. for two hours gave 75% and 69.7% for early and late cultures, respectively, of *L. fermentum*; similar cultures of *L. casei* yielded 83.1% and 78.3% loss.

The effects produced by *Euglena gracilis* on the osmotic pressure of the medium were determined in the following experiment. Since the observed changes in volume of Protozoa and bacteria presumably depend upon a loss or gain of water by the organisms, it was assumed that, if initial expansion of cells from a culture at maximal density results from the synthesis and accumulation of molecules within the cell, the intro-

duction of a sufficient number of cells should appreciably reduce the osmotic pressure of the suspending medium through the uptake of substrates. Accordingly, flagellates from cultures at maximal density in medium A and medium B were resuspended in the homologous media in concentrations of about 500,000 flagellates per ml. Determinations of osmotic pressure were made on the supernatant media by measurement of freezing point depression, using the conventional Beckmann technique. Figure 10 shows that an initial increase of about

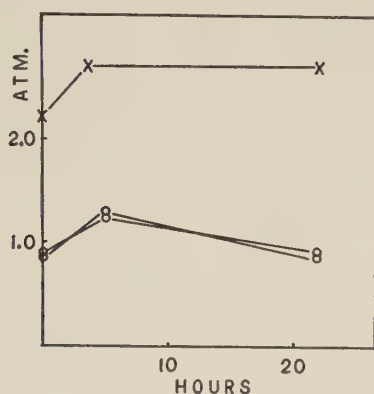


Fig. 10 Alterations of osmotic pressure of media by *Euglena gracilis* at 9°C. Medium A is represented by X, and medium B is designated by a circle.

0.25 atmosphere occurred in both fluids, and medium B returned to the osmotic pressure of the uninoculated medium within 22 hours. The findings were considered an indication of reduced synthesis by virtue of low temperature, although it is noteworthy that, in such experiments, removal of substrates may have been masked to a small extent by the simultaneous removal of water by the flagellates.

DISCUSSION

The Protozoa and bacteria employed in the present study showed either contraction and reexpansion or initial expansion, depending on the growth phase of the parent cultures,

when cells were resuspended in fresh homologous media and maintained at 9°C. to prevent reproduction. When they were taken from a culture in the early logarithmic growth phase, the various organisms underwent an initial contraction, followed by expansion to approximately the initial volume; cells from cultures in the phase of maximum density showed initial expansion. This behavior was observed in *Euglena gracilis*, *Chilomonas paramecium*, *Escherichia coli mutabilis*, *Lactobacillus fermentum*, *L. leichmannii*, and *Streptococcus faecalis*.

Numerous previous workers have concluded that bacteria transferred from cultures in the early logarithmic phase have a lowered capacity to form various enzymes, while those from the phase of maximum density show high enzyme productivity. During the lag phase after transfer of *E. coli* from senescent cultures in peptone medium, a peak in CO₂ and NH₃ production has been reported (Walker *et al.* '34), the rates of production at two hours rising to three times and 5 times those at one hour, respectively. A sharp decline in production of these compounds was noted with the onset of the logarithmic phase. This finding was later corroborated (Clifton, '37). It was further shown (Wooldridge and Glass, '37) that glutamic acid, alanine, and lactic acid dehydrogenases reached maximum activity in older cultures, which were nearing the phase of maximum density. Comparable variation has been reported for *Clostridium welchii* and *C. histolyticum* by Gale ('43), who rejected changes in cellular permeability as an explanation of cyclic variation in enzyme production because such enzymatic changes occurred regularly with different enzymes and in different species. In *E. coli*, production of β -galactosidase was minimal in the early logarithmic phase and maximal in the phase of maximum population density (Porter, Holmes, and Crocker, '53). Among Protozoa, *Tetrahymena geleii* W has shown increased esterase activity in older cultures (Fennell and Marzke, '54), and phosphatase activity of this species is at a minimum in logarithmic cultures (Elliott and Hunter, '51). In the present experiments, the occurrence of contraction and reexpansion in cells from the early logarithmic phase

parallels the reported low enzyme production, and the initial expansion of cells from the maximum density phase might be correlated with the reportedly high enzymatic activity of such cells.

Collectively, the available evidence supports a hypothesis that the initial contraction, which occurs when cells are removed from previously conditioned medium (early logarithmic culture) to fresh medium, may be due to a reduction in number of particles passing inward and the consequent loss of water osmotically. Reexpansion would result from osmotic passage inward of water, following the eventual formation and action of enzymes on substrates to produce cell-penetrating particles. This theory is supported by the writer's data.

It has been shown previously (Wainwright, '53a; '53b) that α -methylglucoside, in dilutions as high as 0.004 M, inhibits the adaptation of *E. coli* in a medium containing no carbon source other than lactose. In the present experiments (fig. 4), organisms from a maximal density culture in dextrose-synthetic medium showed initial expansion when transferred to fresh homologous medium and an initial contraction with subsequent reexpansion in lactose-synthetic medium. In the presence of α -methylglucoside (fig. 5), both the initial expansion in dextrose-synthetic and the reexpansion after contraction in lactose-synthetic medium were prevented. It seems reasonable to correlate this effect of α -methylglucoside on cellular expansion with its inhibitory effect on enzyme production.

In the case of *Tetrahymena pyriformis*, substitution of ornithine for arginine in medium C (fig. 3) induced initial shrinkage and reexpansion of ciliates transferred from a maximal density culture in medium C. In the homologous medium, the organisms showed only an initial expansion. Similarly, *Euglena gracilis* underwent a pronounced shrinkage upon transfer from a peptone medium (medium B) to a synthetic medium (medium A). The occurrence of shrinkage under these conditions is perhaps to be correlated with the absence of or a delay in production of certain enzymes. The data on rate of reexpansion in *Euglena gracilis* at different temperatures (fig. 8) also

may be interpreted on such a basis. It is noteworthy that the slope of the curve diminishes above 26°C., which is optimum for growth of the flagellate in the peptone medium employed. The shape of the curve is typical of that for many enzymes within the physiological range. The favorable effects of higher substrate concentrations on expansion of *E. gracilis* in peptone and synthetic media (fig. 9) might be attributed to the influence of increased substrate on the rate of reaction between enzyme and substrate.

In summary, the writer's data demonstrate that the phenomenon of contraction and reexpansion is influenced by α -methylglucoside, by changes in temperature, and by substrate concentration in the manner expected of an enzyme-controlled process.

The relative effectiveness of dextrose and lactose in promoting expansion of *Escherichia coli* furnished quantitative data supporting the suggested mechanism. Resting, sugar-depleted bacteria gained volume rapidly in 0.004 M lactose, but those in the same molar concentration of dextrose contracted (fig. 5). It may be assumed that, if the disaccharide gave rise to more numerous penetrating particles per mole than did the monosaccharide, the amount of water passing inward across the membrane would be greater in the presence of the disaccharide.

In the case of *E. coli mutabilis* (fig. 6), the lactose-negative variant, in transfers from cultures at different growth phases in dextrose-synthetic medium, failed to attain equilibrium volumes in lactose medium comparable to those of controls in the dextrose. It has been claimed by previous workers (Monod and Audureau, '46; Ryan, '52) that adaptation to lactose involves a selection and multiplication of spontaneously occurring mutants of this strain. In the present experiments, appreciable multiplication was prevented by storage of suspensions at 9°C. Hence these data were considered both a further indication of the involvement of enzymes and controlling genes in contraction and expansion and, also, an independent corrob-

oration of the claim for mutation, which has been questioned by some authors (Dean and Hinshelwood, '54).

In addition to the observation that lactose and dextrose in dilute concentration differ in ability to promote expansion of sugar-depleted organisms, certain other data indicate a dependence of expansion on the availability of substrates which are qualitatively as well as quantitatively adequate to maintain metabolism. Thus the mere restoration of sodium acetate to supernatant culture fluid from a maximum-density culture of *Euglena gracilis* in synthetic medium gave rise to a contraction, rather than expansion, indicating that the net result was an increase of extracellular solute without perceptible passage of particles into the cells. This finding suggested that such factors as pH and oxidation-reduction potential also play a role, as might be expected in view of their influence on enzyme activity. It would seem that uptake of water by the cells is dependent upon *availability* of particles from the external substrate. Lockhart and Powelson ('54) have indicated the importance of this factor in growth of bacteria as a result of their finding that the limiting substrate was not exhausted at the termination of multiplication of *E. coli*. That the presence of adequate amounts of growth-factors also is necessary for cellular expansion was indicated by the failure of vitamin B₁₂-depleted *Euglena gracilis* to undergo expansion in the absence of this vitamin, whereas the expected expansion occurred with an adequate concentration of B₁₂.

Many previous investigators have reported a marked increase in volume of bacteria and Protozoa in very young cultures incubated at temperatures suitable for growth and multiplication. In these cases, synthesis of protoplasmic constituents was favored, whereas, in the present experiments, the low temperature of storage precluded synthesis at normal rates. Thus, the previous reports have described volume increases which were of greater magnitude than those observed in the present work. Larger cells in young bacterial cultures have been reported for 37 different species (Clark and Ruehl, '19), and more recently for *E. coli* (Walker, *et al*, '34; Wade,

'52). A possible influence of cell size on metabolic activity of bacteria has been suggested (Clifton, '37). Similarly, a decrease in size of *Tetrahymena geleii* W in older cultures has been reported (Ormsbee, '42), and an increase in size of this species has been observed (Loefer, '52) in cultures 24–36 hours old, corresponding to relatively young populations. In the present experiments, a decrease in average cell volume with aging of the culture has been observed in *Euglena gracilis* (fig. 2). The composite data support a view that the larger bacteria and Protozoa characteristic of very young cultures gradually encounter a medium in which substrates are depleted and/or less available because of altered pH, etc. At temperatures suitable for growth, the consequent reduction in number of particles entering the cell, with resultant osmotic loss of water, may partially explain the simultaneous reduction in volume. This assumption seems to be supported by the present finding that *E. gracilis* from older cultures, after suspension in fresh medium, expanded at 9°C. without indications of extensive protoplasmic synthesis. Thus, the data on contraction and reexpansion or initial expansion by cells in fresh media provide a possible basis for the explanation of cyclic changes in volume during the growth of cultures.

It is noteworthy that, when organisms underwent initial contraction and reexpansion in the present experiments, the volumes subsequently attained at equilibrium were essentially equal to the initial volumes. This finding was interpreted as evidence that the phenomenon was not a result of protoplasmic synthesis. In the case of *E. gracilis*, a decreased rate of synthesis at 9°C. was indicated by the finding that heavy suspensions of the flagellate did not reduce the osmotic pressure of media appreciably (fig. 10), whereas it might be expected that synthesis of high-molecular-weight molecules at a normal rate would have caused a marked reduction in the number of smaller extracellular particles. For the bacteria studied, the temperature of storage also was well below the optimum growth range, presumably inducing a similar retardation of protoplasmic synthesis in these experiments. Data on *Euglena*

gracilis (table 6), showing the consistently higher density of contracted cells and of those from older cultures and the lower density of cells after reexpansion or initial expansion would seem to indicate the loss and gain of water, respectively. The suggested loss of water by cells with aging of cultures was supported by the finding (table 7) of relatively high cellular density in the maximal density phase for different bacteria and Protozoa, and of lower loss in weight on drying for *Lactobacillus fermentum* and *L. casei* from cultures in this growth phase. It is reasonable to assume that reduced enzymatic activity in early logarithmic cells resulted in a reduced rate of particle entry from the substrate supply in fresh media, causing extracellular passage of water as described for the application of slowly-penetrating solutes (Jacobs, '35). Subsequent intracellular passage of water would occur with increased enzyme formation and enzymatic production of penetrating particles, or with active absorption dependent upon enzymes. Likewise, initial expansion of maximum density cells could result from the entrance of water with particles arising from the action of abundant enzymes on the adequate supply of substrates in fresh medium. Finally, such variation in water content, which occurs also during the growth cycle of bacterial and protozoan cultures, may provide a basis for the cyclic variation in capacity to produce enzymes. In this instance, loss of water might result from reduction in the rate of particle entry due to the reduced availability of substrates. Consequent increase in intracellular concentrations of certain non-penetrating protoplasmic constituents may alter biochemical reactions in accordance with the law of mass action. A possible case of such alteration has been reported (Corbett, '54) for *Streptococcus faecalis*, wherein increased osmotic pressure of the culture medium produced greater growth promotion by folic acid in suboptimal concentrations.

SUMMARY

Euglena gracilis, *Chilomonas paramecium*, *Tetrahymena pyriformis*, *Escherichia coli mutabilis*, *E. coli communis*

Streptococcus faecalis, *Lactobacillus fermentum*, and *L. leichmannii* underwent either contraction and reexpansion or initial expansion in fresh homologous media, depending on the growth phase of the culture from which they were transferred. Contraction and reexpansion were characteristic of organisms from the early logarithmic phase, while initial expansion was found in cells from the phase of maximum density, which reportedly have higher enzyme-producing capacity. Since these observations were made on organisms stored at 9°C., no appreciable cell division occurred, and the data indicate that the observed degree of expansion could not be attributed to protoplasmic synthesis and storage of metabolites. The evidence suggests that the changes in cell volume resulted from a gain or loss of water accompanying fluctuations in the rate of particle entry, which may be correlated with differences in enzyme-producing capacities.

The data on gain and loss of water in the volume changes following transfer of cells to fresh media are in conformity with previously reported decreases in bacterial and protozoan volumes with the aging of cultures. In such cyclic decreases in volume, a lowered rate of particle entry would be expected as a result of less available substrates.

A requirement for induced enzyme formation was found to give rise to a typical, temporary contraction in *E. coli communis*, *Tetrahymena pyriformis*, and *Euglena gracilis* transferred from cultures at maximum population density; organisms of the same types always underwent initial expansion in transfers to fresh homologous media. In contrast, *E. coli mutabilis* (lactose-negative) from cultures in dextrose-synthetic medium failed to attain equilibrium volumes in lactose which were comparable to those reached in dextrose controls. These findings support a previous claim that the selection of spontaneous mutants is a prerequisite to growth after this change of media.

The evidence suggests that progressive loss of water with aging of cultures may be causally related to the cyclic variations in capacity to produce enzymes. Non-penetrating pro-

toplasmic molecules may thus become more concentrated within the cell, causing a shift in equilibrium of biochemical reactions.

ACKNOWLEDGEMENT

The author wishes to express sincere appreciation to Professor R. P. Hall of the Graduate Faculty, Department of Biology, New York University, for his expert and generous guidance in the performance of these experiments and the preparation of the present manuscript.

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AUDITORY RESPONSES IN THE SPECTACLED CAIMAN ¹

ERNEST GLEN WEVER AND JACK A. VERNON
Princeton University, Princeton, New Jersey

FOUR FIGURES

Three studies have dealt with the auditory capacities of members of the crocodile family. Berger in 1924, in an investigation concerned primarily with hearing in lizards, made observations on a single specimen of the blunt crocodile, *Osteolaemus tetraspis*, and found a speeding up of the breathing rate to occur in response to a number of sounds, such as those produced by firing a pistol, ringing a bell, and blowing a whistle. Adrian, Craik, and Sturdy in 1938 used the electrical potential method in a study of hearing in six young alligators 12 to 18 inches in length, and obtained both cochlear and nerve potentials in response to a wide range of tones. The nerve potentials were synchronized with the stimulus waves for frequencies up to 250 cycles. The cochlear potentials were present over a range from 50 to 4000 cycles, and were strongest between 400 and 1000 cycles. Beach in 1944, in a study of five specimens of *Alligator mississippiensis* estimated as up to 5 years old, obtained vocal responses of either roaring or hissing on the plucking of a metal rod or the intermittent blowing of a horn at a frequency of 57 cycles. Less often these responses occurred also to a tone an octave higher. No other tones gave roaring, but others up to 341 cycles gave hissing and head movements. These tests failed to give any indications of hearing in four other alligators of

¹ From the Princeton Psychological Laboratory. These experiments were aided by a contract, N6-onr-270(3), with the Office of Naval Research and by Higgins funds allotted to Princeton University. Permission is granted for reproduction and use by the United States Government.

this species and also in one *Alligator chinensis* and one *Crocodile acutus*.

In the present study the electrical potential method was used to investigate the auditory capacities of young specimens of the spectacled caiman, *Caiman sclerops*. These specimens varied from 16 to 20 inches in length.

The ear of the caiman, in common with others of the crocodile family, has a number of peculiar features. It is the most highly developed of all the reptilian ears, and contains an elongated, curved cochlea closely similar to that of birds. The middle ear cavity is of complex form, and the right and left cavities are interconnected by an air passage that runs transversely across the head dorsal to the brain. The tympanic membrane lies at the end of a shallow auditory meatus, and is protected externally by a pair of ear lids, a large upper lid and a much smaller lower lid that meets the upper one for only about a quarter of its length at the forward end. When the animal is on land the upper lid is usually raised so as to open a nearly horizontal slit that serves for the entrance of sounds. When the animal enters the water and dives below the surface the two lids are tightly closed. The lids are sometimes closed also when the head is out of the water, especially if the animal has been disturbed in some manner, but usually under these conditions the lower lid is pulled down to open a small slit at the forward end, which likewise serves adequately for the entrance of sounds.

EXPERIMENTAL PROCEDURE

For the recording of cochlear potentials the animals were anesthetized with an intraperitoneal injection of ethyl carbamate in reptilian Ringer's solution, and were then operated upon by drilling a hole through the parietal bone into the transverse passage, thereby exposing both tympanic cavities. By looking through this hole in a lateral and ventral direction the round window of either side could be seen. A platinum foil electrode was placed in contact with the round window membrane on one side, usually the left. In most of the experi-

ments the dorsal opening was then sealed with wax, with care not to disturb the electrode wire. An indifferent electrode was inserted beneath the skin of the head. A metal clip was applied to keep the upper ear lid raised (for it is commonly closed in the anesthetized animal), and a sound tube connected with a loudspeaker was sealed over the opening. This sound tube carried a probe tube connected to a condenser microphone for the measurement of the intensities of the applied sounds. Pure tones over a wide range of frequencies were used, and the resulting cochlear potentials were measured with a selective voltmeter (a General Radio Type 736-A wave analyzer). These measurements were carried out in eight animals.

RESULTS

Cochlear potentials were obtained for a fairly wide range of frequencies, from 20 to 6000 cycles, as shown for two different animals in figure 1. This figure indicates the sound pressures required at various frequencies over this range in order to produce a potential of one microvolt. It is evident

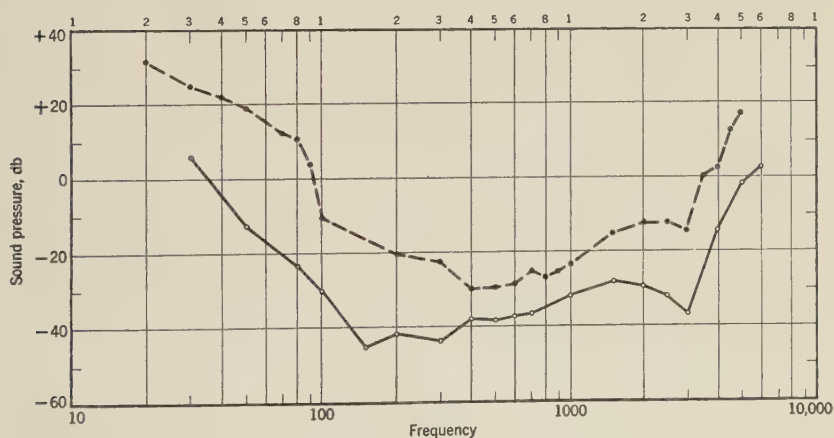


Fig. 1 Sensitivity of the caiman's ear as measured by its cochlear potentials. Curves are given for two different animals. Each curve represents the sound pressure required at various frequencies to produce a standard response of one microvolt. The sound pressure is given in decibels relative to one dyne per cm^2 .

that the sensitivity is greatest in a region from 100 to 3000 cycles and falls off rapidly for lower and higher tones. There were moderate variations in sensitivity among the animals, and the solid-lined curve represents one of the more sensitive whereas the dashed curve represents one of the less sensitive. Frequencies above 6000 cycles were tried, but with them it

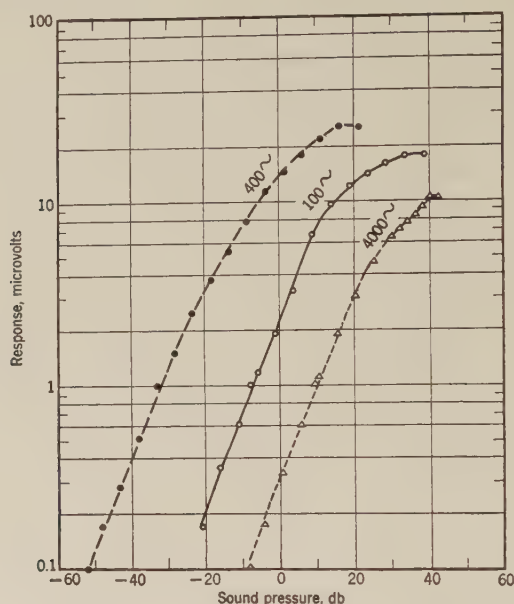


Fig. 2 Intensity functions for three tones. On the abscissa, zero db represents a sound pressure of one dyne per cm^2 .

usually was not possible to obtain measurable potentials at any intensities that were safe to deliver to the ear.

The functional relation between the cochlear potentials and sound intensity is of the same form as that found in all other species of animals that have been studied in this manner. These potentials increase linearly with sound pressure at low levels, and then at high levels they depart from linearity and pass through a maximum, as shown in figure 2 for some representative tones. For sounds in the region of the maximum the ear is endangered, and prolonged

exposure to such sounds produces a serious loss of potentials. In this animal the maximum potentials varied from 10 to 30 microvolts for different stimulus frequencies, in another animal they varied from 11 to 41 microvolts, and in a third animal from 14 to 58 microvolts. In general the maximums had the largest values in the region of 200 to 700 cycles, and declined rapidly as the frequency was raised.

There is no doubt that the ear lids are closed to keep water out of the external ear canal when the animal submerges. We made a number of measurements to ascertain the effects

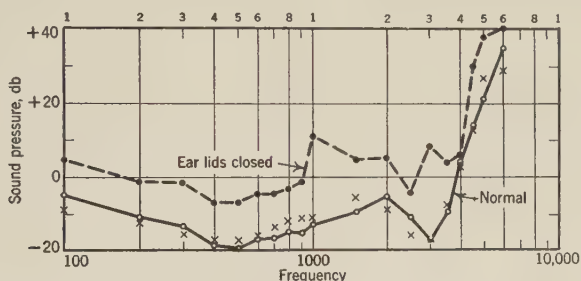


Fig. 3 The sensitivity is shown for three conditions. The solid line represents the normal condition, with the ear lids open. The broken line shows the effects of closing these lids. The crosses indicate the sound pressures required to produce the standard response when the sounds were applied to the opposite ear.

of such closure upon the reception of aerial sounds. Figure 3 gives some of the results. The solid-lined curve represents the sensitivity under normal conditions, when the lids were open; and as usual this curve indicates the sound pressure required over the range from 100 to 6000 cycles to produce a standard response of one microvolt. The ear lids were then closed, and another equal-response curve was obtained for this condition as shown by the dashed curve. In general, the closure of the lids reduces the transmission of aerial sounds by 10 to 12 db, though with some irregularities in the high frequencies. Results like these were obtained only when the lids were firmly closed; as might be expected, the opening of even a tiny crack resulted in practically normal transmission.

Because the two tympanic cavities are interconnected it was of interest to discover the extent to which sounds introduced into one ear were conducted through the transverse passage to the other ear. With the recording electrode on the left round window membrane the stimulating tones were applied first to the left ear in the usual way and then to the right ear. The open circles of figure 3, through which the solid curve is drawn, represent the first condition, and the crosses in this figure represent the second condition; thus the differences between circles and crosses show the effects of sound conduction from one ear to the other. These differences

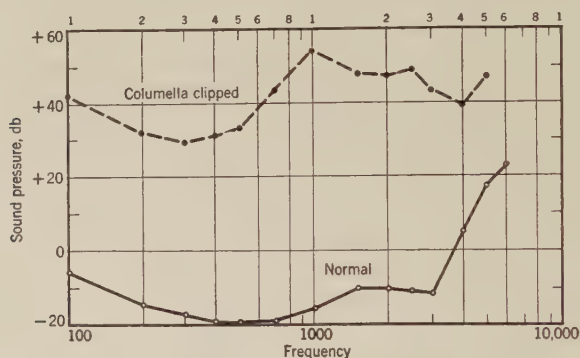


Fig. 4 The effects on sensitivity of clipping the columella.

are small and vary in an irregular manner over the frequency range; their algebraic mean is 0.2 db. It is clear that a sound striking one ear stimulates the other about equally well. This situation raises an interesting question as to the ability of this animal to localize sounds. It would seem that binaural intensity cues can be of little service in such localization. The possibility remains that there may be phase differences in the stimulation of the two ears, which perhaps may operate for this purpose.

Like most other reptiles, the caiman has a simple columellar system that connects the tympanic membrane with the oval window of the cochlea. Figure 4 shows the effects of severing this connection by clipping out a small piece of the

columella. The solid curve of this figure represents the normal condition, when the columella was intact, and the dashed curve represents the sensitivity after the clipping had been carried out. As is evident, this procedure caused a profound loss of sensitivity, amounting to 47 to 60 db for most tones, rising to its greatest value of 70 db at 1000 cycles, and then declining to 30 db at 5000 cycles. After the clipping it was no longer possible to obtain a measurement at 6000 cycles. The mean loss over the range from 100 to 5000 cycles was 52 db. It is evident that the middle ear mechanism is of great service in the transmission of sounds to the inner ear.

SUMMARY

Cochlear potentials were obtained in the spectacled caiman, *Caiman sclerops*, for tones over a range of 20 to 6000 cycles. The greatest sensitivity as measured by these potentials was in the region of 100 to 3000 cycles. The external ear is equipped with ear lids, evidently to keep water out of the auditory canal when the head is submerged, and the closure of these lids reduces the conduction of aerial sound to the ear by 10 to 12 db over most of the frequency range. Because the two middle ear cavities are interconnected, the application of sounds to one ear stimulates the other ear almost equally well. Though the conductive mechanism of the ear is of the simple columellar form as found throughout the amphibians, reptiles, and birds, it is highly effective in the transmission of sound, and the loss of this mechanism produces a profound impairment of sensitivity.

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ENZYME SYSTEMS OF COLPODA CUCULLUS

I. OXIDATION OF CERTAIN KREBS CYCLE INTERMEDIATES

NORVELL W. HUNTER AND ROY HUNTER, JR.

Department of Biology, Morgan State College, Baltimore, Maryland

The genus *Colpoda* has been widely investigated with reference to inorganic and organic compounds that play important roles in the life of the organisms. However, the literature yields little information concerning the specific substances that can be utilized as a source of energy. Since the protozoa are known to differ widely in their ability to oxidize certain components of the tricarboxylic acid cycle, it would seem that an investigation in this respect of *Colpoda cucullus* would be of some interest. Therefore, the purpose of the present communication is to show that (1) certain Krebs cycle intermediates can be oxidized by *C. cucullus*, and (2) certain reactions on the cycle can be blocked by metabolic inhibitors.

MATERIALS AND METHODS

A clone free from other protozoa was established for *C. cucullus*. Stock cultures were carried at room temperature in semi-closed museum jars of 400 ml capacity and containing 100 ml of bermuda grass-alfalfa hay infusion. Separate infusions were prepared for alfalfa hay and dried bermuda grass. The alfalfa hay infusion was prepared in the usual way. The bermuda grass infusion was prepared by boiling 1.5 gm of grass in 1.0 l of tank distilled water for 10 min. After cooling, the total volume was brought back to 1.0 l by the addition of water. Both infusions were stored at 3° C. until used. Immediately before use, the grass infusion was mixed with the hay infusion in a ratio of 2:1, respectively. Animals were harvested from the stock cultures during the logarithmic growth

phase and exposed to 1000 units of penicillin/ml infusion for 12 to 14 hr. prior to being used in the experiments.

Counting of the organisms was accomplished by use of a hemocytometer. Approximately 100,000 organisms per respirometer flask were used for each enzyme determination. Manometric measurements were carried out in Warburg flasks at a temperature of 30°C. The shaking rate was 120/min through a radius of 4.0 cm. Two-tenths milliliters of 10% KOH with a folded piece of starch free filter paper was used in the center well as a CO₂ absorbent. All chemicals used were from commercial sources and of reagent grade.

Cytochrome c. The complete reaction system was a modification of that of Umbreit, Burris, and Stauffer ('51). The system contained 1.0 ml of 0.2 *M* phosphate buffer of pH 7.6, 0.5 ml of 2.4×10^{-3} *M* cytochrome c, 0.2 ml of 0.144 *M* sodium ascorbate, 0.2 ml of 4×10^{-3} *M* aluminum chloride, 1.0 ml of protozoan suspension, and distilled water to a total volume of 3.0 ml. Umbreit, Burris, and Stauffer ('45) published the method used for studying cyanide inhibition (10^{-3} *M*) as used in these experiments.

Succinate. Umbreit, Burris, and Stauffer ('51) published the method used in these studies for the determination of succinate oxidation. The only modification of their method was the use of 1.0 ml of whole protozoan cells, 0.2 ml of aluminum chloride, and 0.2 ml of calcium chloride. 10^{-3} *M* sodium malonate (prepared by neutralizing malonic acid with 1.0 *N* NaOH) was used as the metabolic competitor of succinate for succinic dehydrogenase.

Oxalacetate. Umbreit, Burris, and Stauffer ('51) outlined the method used for determination of the oxidation of oxalacetate. Instead of homogenates, however, 1.0 ml of whole cells was used in these experiments.

alpha-Ketoglutarate. Umbreit, Burris, and Stauffer ('51) published the methods for determination of the oxidation of this substrate. The only modification of their method was the use of 1.0 ml of whole cells.

Malate. Sodium malate was prepared by neutralizing malic acid with 1.0 *N* NaOH. Umbreit, Burris, and Stauffer ('51) published the method used for the enzyme determination. The modification of their method consisted of using 1.0 ml of protozoan cells and 0.023 *M* of sodium malate. The total volume was made up to 3.0 ml.

Fumarate. Sodium fumarate was prepared by bringing fumaric acid to pH 7.0 with 1.0 *N* NaOH. The reaction system contained 0.033 *M* of phosphate buffer of pH 7.6, 0.025 *M* sodium fumarate, 1.0 ml of protozoan cells, and distilled water to a total volume of 3.0 ml. 10^{-3} *M* sodium iodoacetate (prepared by neutralizing iodoacetic acid with 1.0 *N* NaOH) was added to the reaction system to study the inhibition of fumarase. 10^{-3} *M* sodium fluoride was also used as an inhibitor of enzymatic activity.

Citrate. The reaction system was the same as for fumarate except 0.05 *M* sodium citrate was used as substrate.

Glycerophosphate. Read ('53) published the method used for determination of the oxidation of glycerophosphate. The only modification of his work was the use of 1.0 ml of protozoan cell suspension instead of the homogenate.

Glutamate. The reaction system used for studying the oxidation of this substrate was the same as for fumarate, except 0.05 *M* monosodium glutamate was used as the substrate.

RESULTS

Table 1 summarizes the average results of numerous experiments utilizing various substrates that lie on or near the Krebs cycle. In all cases, the endogenous respiration has been subtracted from the values. The table indicates that cytochrome c was oxidized at a much greater rate than the other substrates. The table shows further that the descending rate of oxidation of the substrates investigated was as follows: cytochrome c, succinate, oxalacetate, alpha-ketoglutarate, malate, citrate, fumarate, glycerophosphate, and glutamate. Too, there is indication that alpha-ketoglutarate and oxalacetate were oxi-

dized at about the same rate. This was also true for glutamate and glycerophosphate.

10^{-3} M cyanide caused approximately 70% inhibition of oxygen uptake with cytochrome c as the substrate. 10^{-3} M malonate inhibited the oxygen uptake with succinate as the substrate by an average of 40%. Fumarate uptake was inhibited approximately 65% by 10^{-3} M iodoacetate and approximately 65% by 10^{-3} M fluoride.

TABLE 1

Oxidation of some Krebs cycle intermediates by Colpoda cucullus

| SUBSTRATE | AVERAGE μO_2 UPTAKE/ 100,000 ANIMALS/HR. ¹ | σ ² |
|-------------------------------|---|-----------------------|
| Cytochrome c | 175.1 | 3.5100 |
| Succinate | 14.3 | 2.6961 |
| Oxalacetate | 12.7 | 1.3132 |
| alpha-Ketoglutarate | 12.0 | 0.9654 |
| Malate | 10.1 | 0.6552 |
| Citrate | 8.2 | 0.3576 |
| Fumarate | 8.2 | 0.6973 |
| Glycerophosphate ³ | 6.8 | 1.0240 |
| Glutamate ³ | 4.8 | 0.3641 |

¹ Values corrected for endogenous respiration.

² Standard deviation.

³ Substrates included because of their proximity to the Krebs cycle.

DISCUSSION

A mixture of bermuda grass and alfalfa hay infusions makes an excellent culture medium for *C. cucullus*. The animals grow more rapidly and show less tendency to encyst than with alfalfa hay infusion alone. Furthermore, the mixed infusion causes rapid excystment of encysted forms. Bacteriological tests indicate that penicillin used at the levels of 1000 units/ml of infusion is ample for sterilization within the time limits of these experiments. At this concentration the penicillin does not cause a deleterious effect on the protozoans as judged by the motility of the animals.

Umbreit, Burris, and Stauffer ('51) point out that cytochrome oxidase is found considerably in excess to succinic dehydrogenase in rat liver cells. Such an observation seems to be true in *C. cucullus* because our findings indicate that cytochrome c is oxidized at a much greater rate as compared with the other substrates investigated. In this protozoan, cyanide has no immediate effect on the cytochrome system; however, 20 minutes after the initial manometric readings, a sharp decline occurs in the oxygen uptake by *C. cucullus*.

Succinate appears to be oxidized at a slightly greater rate than oxalacetate and alpha-ketoglutarate. This small difference may be due to a number of optimum factors which were not investigated, such as the pH of the reaction system, final concentration of substrate, enzyme and/or co-factors, and other factors such as rate of substrate penetration of the cells. The depression of the oxygen uptake by malonate when succinate is the substrate is well known for many other animal cells. Our findings for *C. cucullus* in this respect are in agreement. Malate appears to be oxidized at a somewhat greater rate than fumarate and citrate. A close parallelism seems to exist between the oxidative rates of the latter two substrates. The table indicates that there is no considerable variation in the rates of oxidation of three of the C-4 substrates (succinate, fumarate, and malate) but that oxalacetate is much more rapidly oxidized. The previous explanation suggested concerning optimum factors may well apply to these variations in the oxidation of the C-4 substrates mentioned.

The glycerophosphate oxidizing enzyme in *C. cucullus* appears to be cytochrome-linked. This conclusion is based on the fact that when homogenates of the potozoans are used together with diphosphopyridine nucleotide (DPN) in the glycerophosphate medium, no oxygen uptake occurs above the endogenous rate. This, however, is not the case when cytochrome c is substituted for the DPN. Glutamate apparently is oxidized more slowly than any of the other substrates investigated. As slow as this rate appears, nevertheless, it suggests

at least one of the possible pathways for amino acids entering the Krebs cycle of these protozoans.

SUMMARY

1. *Colpoda cucullus* possesses enzyme systems capable of oxidizing substrates investigated in the following descending order of rapidity: cytochrome c, succinate, oxalacetate, alpha-ketoglutarate, malate, citrate, fumarate, glycerophosphate, and glutamate.

2. Cyanide inhibits the oxygen uptake with cytochrome c as the substrate; malonate inhibits succinate oxidation, and oxygen uptake with fumarate as the substrate is substantially decreased by both fluoride ions and iodoacetate.

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SUPPLEMENT

SOCIETY OF GENERAL PHYSIOLOGISTS

Abstracts of papers presented at the twelfth Annual Meeting ¹, Marine Biological Laboratory, Woods Hole, Massachusetts, September 3, 4, and 5, 1957.

¹ Abstracts or summaries of programs of previous meetings will be found in Biol. Bull., 91: 236; 93: 222; 95: 281; 97: 267; 99: 308 (1946-50); Science, 114: 699 (1951) and 118: 768 (1953); J. Nat. Cancer Inst., 13: 1399 (1953); and J. Cell. and Comp. Physiol., 44: 327; 46: 353; 48: 329 (1954-56).

ABSTRACTS

1. PACEMAKER ACTIVITY ANALYZED THROUGH OSTERHOUT'S NITELLA EXPERIMENT.¹ Reinhard H. Beutner, Des Moines Still College, Des Moines, Iowa.

The mechanism of a pacemaker can be analyzed through Osterhout's experiments on the conducting protoplasm of the fresh water plant *Nitella*. In contact with tap water or dilute KCl (0.001 M.) the electric potential difference of the protoplasm of this plant is positive on the outside, but turns toward negative upon contact with more concentrated KCl (0.01 M.) solutions. An area of the protoplasm, thus rendered negative, sends out rhythmical waves over the still untreated portion of the protoplasm, for the following reasons: 1. A local electric circuit arises between the positive and negative p. d. 2. This current elicits electric negativity in the adjacent area through the *transmembrane metabolism* (reversible phospholipid splitting); 3. Subsequently this negativity travels along the nerve fiber as predicted by Lillie's theory. 4. The area initially rendered negative, remains negative for a while, so that a second impulse cannot immediately originate (refractory period). 5. An oxidative process of recovery restores the positive potential negative. 6. Another electrical circuit is thus formed. This process repeats itself sending forth rhythmical waves. All we have to assume for explaining a natural pacemaker is the existence of a permanently negative area on the nerve membrane surface. A localized permanent negativity may arise because of the local absence of oxidizing enzymes. The requisite conditions for generation of rhythmic impulses are thus present. The indispensable p. d. along the conducting surface is invariably restored by enzymatic oxidation.

¹ Investigation supported by research grant from National Heart Institute, P.H.S.

2. RESPONSE PATTERN AND SUMMATION IN A CRUSTACEAN PHOTORECEPTOR. Donald Kennedy. Syracuse University, Syracuse, N. Y.

Illumination of the caudal abdominal ganglion of the crayfish ventral nerve cord gives rise to impulse trains in a few afferent cord fibers; the activity of a small number of such fibers, or of a single unit, can be recorded from dissected, chilled cords. Such units show, under normal conditions, a prolonged, slowly adapting discharge even to a short flash of light. Under sustained illumination they may fire for up to an hour with only slight frequency changes. At low intensities, the impulse frequency in a single unit rises to a maximum and then declines gradually. At higher intensities, a very fast maximum of discharge is seen, followed by a post-excitatory depression and then a secondary increase. The extent of the inhibition increases with the stimulus intensity. Low external potassium increases the phasic characteristics of the response: it limits after-discharge, raises the threshold, and often causes "burst" firing during a continuous stimulus. Single photoreceptor units show summation in response to

short, subliminal flashes as much as twelve seconds apart; "decay curves" for subliminal excitation have been constructed by experiments in which the spacing of such stimuli is varied. The excitatory process taking place during the long (up to ten seconds) latent period of a prolonged, low-intensity stimulus has also been studied by interposing very short, barely subliminal flashes at various times during the latent period.

3. SOME RECENT, PUBLISHED EXPERIMENTS AND THEIR BEARING ON THE ORIGIN OF BIOELECTRIC FIELDS AND "ACTIVE" TRANSPORT IN THE FROG'S SKIN. E. J. Lund, The Lund Laboratory, Austin, Texas.

Re-examination of the pattern of distribution of bioelectric potentials in the frog's skin, with apparatus and procedure which in so far as electrical measurements are concerned duplicate those by Ussing and Zerahn '50, and are similar to those of Linderholm '52. Others show that there is a non-uniform distribution of potentials in the skin. Potentials in small adjacent areas of the skin may differ by as much as 400%. These patterns of potential are relatively stable.

(1.) In view of this fact it would appear that before any equivalence between the total quantity of electrical energy generated by the skin in this complex field and the coulomb equivalent of ions (Na^+) actively transported by the skin, can not be established by an externally applied current from a battery.

(2.) Since the skin and the battery are operating in series, the energy required for "active" transport must have been partly (or wholly) derived from the battery. Hence in this case the observed approximate coulomb equivalence of ion transport and observed electric current would be expected.

(3.) The complex problem of superposition of polarization imposed by the battery current upon the inherent potential of the skin is also involved.

4. EFFECT OF DINITROPHENOL ON ENZYMATIC ACTIVITY OF MYOSIN B. J. J. Blum and E. Felauer. University of Michigan, Ann Arbor, Michigan.

It has been reported by several workers that 2, 4 dinitrophenol (DNP) accelerates the ATP-ase activity of myosin. Using five hour myosin B and glycerinated *psaos* fibrils, we have measured the (reciprocal) Michaelis-Menten constant, \bar{K} , and the maximum velocity, V , of hydrolysis for ATP, UTP and ITP at 0.3 M KCL, 10^{-3} M Ca, pH 7.0 (TRIS or barbital buffer), 25°C. DNP (10^{-3} M) lowers \bar{K} (ATP) and raises V (ATP) in such a way that the product $\bar{K}V$ remains essentially constant. This is not true at 0.6 M KCL. UTP, with a smaller \bar{K} and larger V than ATP, responds in the same way to 10^{-3} M DNP. ITP-ase activity is not appreciably affected by this concentration of DNP. Preliminary evidence indicates that CTP does not behave like ATP, in that DNP appears to increase \bar{K} and decrease V . The product $KV(\text{CTP})$ is changed upon the addition of DNP. These data are examined with the aid of a previously suggested kinetic scheme based on the idea that there is a slow desorption of ADP from the ATP-ase site. It is possible to explain the constancy of $\bar{K}V$ under certain conditions by use of this kinetic treatment. The possibility that DNP acts by decreasing the binding of the nucleotide ring to the site will be discussed.

5. **MYOGLOBIN DISOXYGENATION IN RELATION TO MUSCULAR ACTIVITY.** Dietrich W. Lübbers and Frans F. Jöbsis, University of Pennsylvania, Philadelphia, Pennsylvania.

The intra-cellular location of myoglobin should, by the application of differential, double-beam spectrophotometry, provide data on the instantaneous changes in the O_2 -consumption during and after muscle contractions (Millikan). We find for the excised turtle skeletal muscle a fast and a slow cycle of absorbancy changes at wave-lengths appropriate to Mgb-Mgb. O_2 in response to contractile activity. For example, the coraco-hyoideus ($26.5^\circ C$, 95% O_2 , + 5% CO_2) when stimulated twice per second for ten seconds, shows first a small absorbancy change which starts during the contractions, attains a maximum a few seconds after the cessation of stimulation, and is followed by a reversal of the absorbancy change. The second cycle is considerably larger and longer, reaching its maximum after approximately five minutes. Assuming that the disoxygenation of Mgb. O_2 is a reflection of the respiratory rate, we conclude that a fast and a slow cycle of aerobic recovery occur in the muscle. Related changes are observed in the cytochromes of Mgb-free muscles (Weber and Chance, Jobsis and Chance). We were also able to make these measurements on the isolated ventricle of the turtle heart, for which purpose medium-sized specimens had to be used as Mgb was not found in the hearts of young turtles. Here, during isometric contractions only a fast cycle was observed. This seems reasonable, considering the normal rhythmicity of this tissue, where metabolic activity should be in a dynamic equilibrium with contractile activity.

6. **MUSCLE STRIATION AND IMAGE ERROR IN POLARIZATION MICROSCOPY** by Shinya Inoué, University of Rochester, Rochester, N. Y.

In conventional polarizing microscopes the light beam is depolarized by the lenses in such a way that the diffraction image of each point in the object is no longer a regular Airy disc. Instead, a point is represented by a clover-leaf pattern which is modified to varying degrees depending on the amount of birefringence of the object. Therefore, the image of a small and/or periodic, weakly birefringent object tends to be spurious and may be quite misleading if taken to represent the exact distribution of birefringence in the object. Such an error will be shown to exist in the striation pattern of muscle fibers.

The depolarization and hence the image error can be eliminated by the recently developed polarization rectifier which also vastly increases the extinction of the polarizing microscope at high numerical apertures.

7. **POTASSIUM RETENTION AND METABOLISM IN TOAD SCIATIC NERVE.** C. Paul Bianchi. National Institutes of Health, Bethesda, Maryland.

Glucose slows the rate of K^+ loss from desheathed *Bufo marinus* sciatic nerve in the first 4 to 6 hours. Thereafter, the beneficial effect of glucose on anaerobic K^+ retention is lost. This is consistent with earlier results on frog sciatic nerve. In addition it has been found that cessation of potassium loss, during a 2 to 3 hour postanoxic recovery period in oxygen, is invariably hampered by prior

anaerobic utilization of glucose for periods as brief as 4 hours. Increase of the phosphate level causes greater loss of K^+ , but during the oxidative recovery phase the rate of loss was no greater in glucose than in the absence of the sugar.

Spike potential measurements showed a decrease which paralleled the rate of loss of K^+ . The spike potential decreased during anoxia, the decrease being substantially slower in the presence of glucose. When oxygen was admitted, the spike potential partially recovered within 10 minutes, the recovery being less in glucose medium.

8. POTASSIUM AND THE CHICK EMBRYO. Evelyn Howard, The Johns Hopkins University¹, Baltimore, Md.

The supply of potassium carried by the hen's egg results in potassium values of the order of 42 m-eq. in the white, and somewhat more in the yolk, amounts well outside the range that would be tolerated in adult plasma. The K in chick embryo plasma and amniotic fluid, sampled at incubation ages of 6 to 18 days, was found to remain between 3 and 5 m-eq. At 2 days, subgerminal fluid potassium was 13 m-eq., considerably lower than unincubated egg white, although the subgerminal fluid is presumably derived largely from egg white. Thus, the early subgerminal fluid prevents the embryo from being directly exposed to the high K levels present in the yolk and white.

Comparisons of K/Na ratios and freezing point depressions in subgerminal fluid and egg white suggest that there may be an active transfer of Na from egg white to subgerminal fluid, mediated by the blastoderm, during the earlier stages of incubation. Water may follow Na osmotically, thus contributing to the characteristically rapid initial increase in the volume of the subgerminal fluid. The subgerminal fluid potassium rises considerably in later stages as the yolk begins to break down, but by this time the mesonephros is presumably able to regulate plasma K levels.

¹ Supported in part by a research grant, No. H-2414, from the United States Public Health Service.

9. SENSITIVITY OF HYDRA TISSUES TO X-RAYS. Helen D. Park, National Institutes of Health, Bethesda, Maryland.

Hydras (*H. littoralis*) in a number of stages of asexual development were irradiated with X-rays at doses of 4,500, 13,000, 25,000 or 30,000 r in a dilute solution containing NaCl, KCl, and CaCl₂. The animals were immediately washed, placed in fresh solution of the same composition, and kept for 1-10 days. The effect of the various exposures on viability, bud initiation and bud differentiation was determined by comparing the number of survivors, the number of animals initiating buds, and the number of buds differentiating completely with nonirradiated controls. Of the three variables studied, viability was least affected by the radiation; the numbers of survivors at 4,500 and 13,000 r were no different from those of the controls. Bud development was slightly inhibited by 13,000 r but some tissue differentiation still took place after 25,000 r. On the other hand, the number of buds *initiated* was greatly reduced at the lowest dose used. In addition, adults were compared with their newly detached young

with respect to ability to reproduce asexually and with respect to viability. It was found that at the lowest dose used the reproductive capacities of the two groups were equally depressed. At all higher doses tested there was no reproduction in either group. Survival in both groups was not influenced at doses of 4,500 or 13,000 r. However, at the 30,000 r level there were almost three times as many young as parent survivors.

10. CELL DEATH AND "UNBALANCED GROWTH" IN *Neurospora*¹. Bernard S. Strauss, Syracuse University, Syracuse, N. Y.

Mutant spores of *Neurospora* incubated on minimal-agar plates show one of two types of behavior. One mutant type will survive a week's incubation on minimal medium and will then respond to the addition of growth substance by the production of colonies. The second mutant type dies after incubation for one or two days being unable to respond to addition of its growth factor (Grigg effect). A vitamin B₆-requiring and an inositol-requiring strain have been studied as examples of the latter category while a methionine-requiring strain has been studied as an example of the former. Strains which die seem to be characterized by the ability to germinate rapidly in minimal medium and to incorporate materials into protein and into material soluble in hot trichloroacetic acid (nucleic acid) at a much greater rate than strains which survive incubation on minimal medium. Strains which kill themselves off do not excrete a toxic substance into the medium. Inhibition of germination by ethionine prolongs the survival of both the vitamin B₆-requiring and inositol-requiring strains. The phenomenon is reminiscent of the cell death due to "unbalanced growth" in the thiamine-requiring mutant of *E. coli* reported by Cohen and Barner. In this case however, cells requiring substances other than nucleic acid constituents display the effect.

¹ Supported in part by funds from the U. S. Atomic Energy Commission and the National Science Foundation.

11. SYNODIC MONTHLY CYCLES IN AN ORGANISM IN CONSTANT ILLUMINATION, TEMPERATURE, HUMIDITY, AND PRESSURE¹. Frank A. Brown, Jr. Northwestern University, Evanston, Illinois.

Five independent, continuous-recording respirometer ensembles, each with four cores of potatoes bearing eyes, were operated for 13 out of the 14 months from Feb. 1, 1956 through Mar. 31, 1957. The respirometers remained sealed and undisturbed for periods ranging from 2 to 12 days (Av. 4.09), being opened and the organisms exposed to room conditions for only 15 to 25 minutes, irregularly at these intervals, for purposes of maintenance of the respirometers. The same individual plants were retained as long as possible (up to several months). Twelve uninterrupted synodic months of hourly data were obtained. Clear average lunar cycles, with a common form, were observed for two lowly intercorrelated phenomena: 1. mean daily rates of O₂-consumption. 2. mean daily acceleration in O₂-consumption following the sealing of the respirometers. For both phenomena, the monthly minimum occurred on the day of the new moon and the maximum fell on the 25th day following new moon. Expressed as per-

centage deviations from synodic monthly means, the range for rate from days n. m. through n. m. + 10 to days n. m. + 17 through n. m. + 26 was $-4.72 \pm 1.27\%$ to $+6.72 \pm 2.27\%$ (Diff. = $11.44 \pm 2.6\%$). For acceleration, from days n. m. - 1 through n. m. + 3 to days n. m. + 22 through n. m. + 26, the range was $-1.78 \pm 0.806\%$ to $+5.17 \pm 2.03\%$ (Diff. = $6.95 \pm 2.18\%$). The last actually involved an average monthly 2.3-fold range in acceleration. Rearrangement of the data for hypothetical 32-day or 28-day cycles, on the other hand, yielded no suggestion of other than total randomness.

¹ These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Northwestern University, NONR-122803.

12. RESPIRATORY ENZYMES OF *Chlamydomonas* MUTANT. Britton Chance and Ruth Sager. University of Pennsylvania, Philadelphia, Pa., and Columbia University, New York, N. Y.

In a pale green mutant of *Chlamydomonas*, steady-state changes caused by illumination are in the direction of oxidation of the respiratory carriers. Anaerobically, pyridine nucleotide and cytochromes of types *b* and *c* are observed to become considerably oxidized. Oxygenation causes oxidation of reduced pyridine nucleotide, flavoprotein, and cytochromes of types *b* and *c*; no absorption bands attributable to cytochromes *a* or *a_s* are observed. Aerobically, illumination of the cell suspension causes a further oxidation of the reduced pyridine nucleotide and of cytochrome *f*. The fact that reduced pyridine nucleotide is oxidized upon illumination of the whole cell suggests that the significance of pyridine nucleotide reduction observed by other workers upon illumination of isolated chloroplasts should be re-evaluated.

The nature of the dark oxidase in these cells is not established; CO produces a very small shift in the prominent absorption bands of the reduced respiratory carriers of the pale green mutant. In recent studies of a new mutant containing considerably less chlorophyll, addition of CO to the reduced carriers causes the appearance of the peak of an absorption band at 416 μ and a trough at 430 μ . These are wavelengths appropriate to the terminal oxidase of several types of non-photosynthetic microorganisms. This pigment is also found in various photosynthetic purple bacteria. The action spectrum for the reversal of CO inhibition of respiration is required for proof of the identification of this pigment with the terminal oxidase of the mutant cells.

13. METABOLIC STUDIES OF PHOTOSENSITIZED RABBIT ERYTHROCYTES. James W. Green. Rutgers University, New Brunswick, N. J.

Saline washed rabbit erythrocytes were suspended in phosphate buffered NaCl in concentrations of 0.5 ml of cells per 100 ml of solution. The fluorescent dye, Rose Bengal, was added to such suspensions in 1 part per million. The experimental suspensions were irradiated for 30 sec at 15° C with a 12 W fluorescent lamp mounted in a condenser-like apparatus. Irradiated and control suspensions were concentrated centrifugally and, after the addition of 1 mg of glucose per ml of suspension, incubated at 35° C for periods up to 10 hours. Glucose disappearance and lactate formation were determined in suspensions of

pH 7.0, 7.2 and 7.4. Preliminary results indicate that a larger amount of glucose disappears from irradiated than from non-irradiated suspensions at pH 7.2 and 7.4 with no certain differences at pH 7.0. By contrast, however, lactate production appears to be greater in the non-irradiated suspensions. The possible significance of these findings and their relation to the cation exchanges accompanying them were discussed.

14. MOLECULAR ASPECTS OF TUMOR-HOST RELATIONSHIPS. Vernon Riley. Sloan-Kettering Institute for Cancer Research and Memorial Center for Cancer and Allied Diseases, New York, N. Y.

If it is assumed that tumors are metabolically or otherwise different from the normal host tissue, it would appear that this distinction would express itself qualitatively or quantitatively in some of the intermediate or terminal biochemical products of malignant growth. A simple experimental design has been employed to demonstrate such differences in animals bearing transplanted tumors of various types in comparison with otherwise identical normal control animals. The principle of the procedure involves the challenging of tumor-bearing and normal mice with an intraperitoneal lethal dose of an appropriate compound capable of reacting *in vivo* with one or more of the unique metabolites associated with the tumor in question. The effect of this reaction on the survival times of the two groups of animals is then determined. Dissimilarity in survival times is presumably due to a decrease or increase in the toxicity of the administered compound which, in turn, is thought to be an expression of its selective binding or chemical alteration by the tumor or its products. This measurable difference, as well as other variations in response, reflects the biochemical differences in the tumor-bearing hosts as contrasted with the controls. The mouse melanoma has been employed as the primary model since some of its aberrant metabolic products are known. For example, PPDA (*p*-phenylenediamine) combines *in vitro* with DOPA (dihydroxyphenylalanine) in an oxygen-consuming, pigment-producing reaction. DOPA is a known component produced in the biochemical chain of events in the metabolism of pigmented melanomas, and thus a rational foundation is provided for an *in vivo* reaction when PPDA is administered to melanoma-bearing animals. The consequence of this procedure, with the above combination, is a significant difference in the mean survival times of the two groups with a protective effect provided by the presence of the tumor. This phenomenon can be inverted by altering the administered compound or by employing a different histological or physiological type of tumor, in which event the tumor may become a biochemical liability to the host.

15. THE ACTION OF THE HERBICIDE, DCMU, ON THE OXYGEN EVOLVING MECHANISM OF PHOTOSYNTHESIS. Norman I. Bishop. University of Chicago (Fels Fund), Chicago, Illinois.

A new herbicide, 3 (3, 4-dichlorophenyl)-1, 1 dimethylurea (DCMU), is such a powerful inhibitor of the reduction of dichlorophenol-indophenol by chloroplasts that, according to Wessels, 2×10^{-7} M is sufficient to cut the reaction rate in half. This concentration was lower than the chlorophyll concentration in

his experiments. The specificity of this new inhibitor for some component of the Hill reaction must be very high.

Further study of the action of this inhibitor was performed on algae adapted for the utilization of hydrogen. Using the green algae *Scenedesmus obliquus* strain D₂ and *Ankistrodesmus braunii* strain Marburg, adapted to an atmosphere of hydrogen-5% CO₂, photosynthesis and photoreduction were studied at different light intensities. Photosynthesis was reduced to one-half by 5×10^{-7} M DCMU, and completely inhibited by 3×10^{-6} M. Inhibition occurred at all light intensities. Photoreduction was not inhibited. Indeed, reversion of photoreduction to photosynthesis at higher light intensities was prevented by 3×10^{-6} M DCMU. This deadaptation did occur, however, in the presence of 5×10^{-7} M DCMU, but at a much higher light intensity than in the control.

Addition of Mn⁺⁺ and Fe⁺⁺ ions, known to be involved in the oxygen producing apparatus of photosynthesis, did not produce any recovery from DCMU poisoning. Manganese-deficient algae have been shown to have decreased photosynthetic activity. Such deficiency primarily affects the oxygen evolving system. Studies on the influence of DCMU on Mn⁺⁺-deficient algae were initiated to determine whether DCMU acts at the same point on the oxygen-evolving pathway as Mn⁺⁺, or at a different site. There was no marked difference between normal and Mn⁺⁺-deficient algae in respect to their behavior toward DCMU.

16. NEW EVIDENCE FOR THE DEPENDENCE OF THE BIOELECTRIC FIELD ON THE CYTOCHROME SYSTEM IN THE ROOT OF *Allium cepa*. E. J. Lund, J. N. Pratley, and H. F. Rosene. The Lund Laboratory, and The University of Texas, Austin, Texas.

It was recently shown that in the absence of oxygen, the concentration of hydrogen donor increased in the root tip cells of *Allium cepa*. This increase could be removed by readmission of oxygen, and corresponded to the rebound of the bioelectric field, Lund and Norris, '55.

When air is replaced by a mixture of 80% CO and 20% O₂, or pure CO the polarity potential is immediately and irreversibly reduced to a small value. If now white light is applied to the region at and between the electrodes, the polarity potential returns immediately to a level usually above (rebound) its normal value and remains.

The nadi reaction is positive and the distribution of its intensity corresponds to the distribution of the magnitude of the polarity potential.

REGULATION OF THE
DISTAL RETINAL PIGMENT OF THE DWARF
CRAWFISH, *CAMBARELLUS*
SHUFELDTI¹

MILTON FINGERMAN

Department of Zoology, Newcomb College, Tulane University,
New Orleans 18, Louisiana

SEVEN FIGURES

Distal retinal pigment of the arthropod compound eye has been termed the "iris pigment" because of its functional similarity to the iris of the vertebrate camera-type eye (Exner, '91). The early literature concerning the mechanics of migration of retinal pigments was reviewed by Parker ('32).

The experiments of Bennitt ('32) first suggested a hormonal control of retinal pigments in the prawn *Palaemonetes vulgaris*. He covered one eye and illuminated the other with the result that the retinal pigments of both eyes assumed a light adapted condition. Kleinholz ('36) demonstrated a light adapting hormone in the eyestalk of *Palaemonetes*. Extracts prepared from eyestalks of light adapted prawns had twice the light adapting potency of eyestalk extracts from dark adapted prawns, suggestive of a role of the hormone in normal regulation of the distal retinal pigment. Eyestalks of the crawfishes *Cambarus bartoni*, *Procambarus clarki*, and *Orconectes limosus*, also contained a factor that light adapted distal retinal pigment (Welsh, '39).

Sandeen and Brown ('52) showed that the position of the distal retinal pigment of *Palaemonetes* between the illuminations of 0.0005 and 50 ft. c. was a function of the intensity of

¹ This investigation was supported by Grant No. B-838 from the National Institutes of Health.

illumination. The brightness of the visual field was the only determinant of the position. Brown, Fingerman and Hines ('52) presented evidence to support the contention that the graded response of the distal retinal pigment demonstrated by Sandeen and Brown ('52) depended upon the quantitative production of a light adapting hormone. Brown, Hines and Fingerman ('52) demonstrated that the central nervous organs of *Palaemonetes* also produced a distal retinal pigment light adapting hormone. These investigators showed in addition that the eyestalks and central nervous organs were also sources of a retinal pigment dark adapting hormone. Through appropriate stimuli of light and darkness secretion of either one of the retinal pigment hormones could be induced (Brown, Webb, and Sandeen, '53).

The present investigation was undertaken to study the regulation of the distal retinal pigment of the dwarf crawfish and to compare the results with information available for other species as a basis for ultimate formulation of a unified view of the physiology of the compound eye.

MATERIALS AND METHODS

Specimens of the dwarf crawfish, *Cambarellus shufeldti*, were collected at Crown Point, Louisiana, for use in these experiments. The stock supply of crawfish was maintained under normal day-night conditions. Experiments were performed between 8 A.M. and 4 P.M. during June and July, 1955.

The method described by Sandeen and Brown ('52) was employed to facilitate rapid determination of the state of the distal retinal pigment in a group of *Cambarellus*. The crawfish were placed one at a time ventral surface down on the stage of a stereoscopic dissecting microscope. With the aid of an ocular micrometer and transmitted light (1) the width of the translucent distal portion of the compound eye in a plane parallel to the long axis of the eyestalk (A in fig. 1), and (2) the length of the eye from the corneal surface to the apex of the notch at the proximal portion of the eye (B in fig. 1) were measured. To render the distal clear portion

of the eye more translucent and the proximal edge of this clear area more definite, the crawfish were submerged in a dish of water on the stage of the microscope. The ratio, A/B , will be referred to as the distal pigment index. Use of this ratio minimized effects of size differences. In the fully dark adapted eye no clear area was present; the distal pigment index was 0.00. In the fully light adapted eye the distal pigment index was 0.10. A typical ratio for a fully light adapted eye was $2/20$.

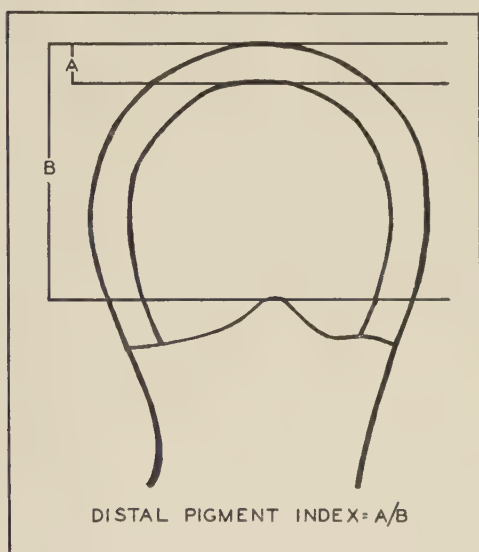


Fig. 1 Diagram of the dorsal surface of the eyestalk of *Cambarellus* to illustrate the method of determining the distal retinal pigment index.

A magnification of $60\times$ was used in the measurements. Each unit of the ocular micrometer at this magnification was equivalent to 24.4μ . The distal pigment index of 10 crawfish could be determined with ease in three minutes. For all experiments the animals were placed in pans with a bottom diameter of 14.5 cm containing aerated tap water one inch deep.

To determine changes that occurred at a series of time intervals after subjecting the crawfish to an experimental

condition as many pans of animals as the experiment required were set up and exposed to the same set of conditions. At each selected interval the crawfish in one pan were assayed and then discarded. In this fashion any effect of handling the animals was eliminated. The smooth trends in the results presented below fully justified this technique.

Extracts of organs were prepared as follows. The organs were excised and placed in van Harreveld's solution until the required number had been removed. They were then transferred to a glass mortar with a minimum of saline and triturated. The organs were resuspended in sufficient van Harreveld's solution to yield the desired concentration. Control animals were injected with van Harreveld's solution. Each crawfish received a dose of 0.02 ml.

Light intensities greater than one ft. c. were measured with a General Electric Photometer; intensities less than one ft. c. were determined by calculations based on the inverse square law. To obtain intensities less than one ft. c. a 25 watt incandescent light bulb was painted black except for an aperture with a diameter of $\frac{1}{4}$ " and was used in the darkroom to avoid stray light. The desired light intensities were obtained by adjusting the distance between the light source and the crawfish.

EXPERIMENTS AND RESULTS

Relationship between intensity of illumination and distal pigment index

A series of illuminations ranging from 0.00174 to 4500 ft. c. was used to determine the influence of intensity of illumination on the position of the distal retinal pigment. The intensities of 3500 and 4500 ft. c. were obtained with sunlight. The remaining intensities were obtained in the laboratory by placing containers of crawfish at appropriate distances from the sources of illumination.

In the first series of experiments 12 *Cambarellus* were put into each of a series of white enameled pans containing water. The pans were then placed under the appropriate illumina-

tions for two hours when the average distal pigment index of 10 animals in each pan was determined. Crawfish were carried individually from the pans to the microscope to assure that no change occurred in the state of the distal pigment due to change in illumination near the microscope. Crawfish were returned to the stock aquaria after the measurements were performed. Water in the pans placed in sunlight was changed at 15 minute intervals to eliminate effects of heat upon the distal retinal pigment.

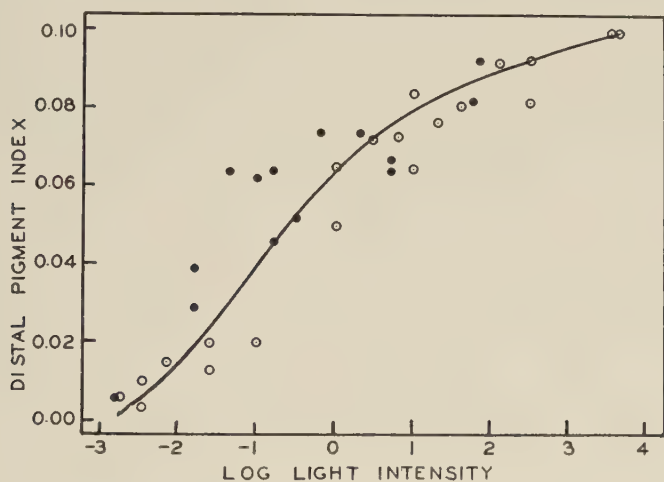


Fig. 2 Relationship between logarithm of light intensity and average distal pigment index. Circles, crawfish on a white background; dots, crawfish on a black background.

In a second series of experiments the average distal pigment indices of *Cambarellus* in black pans at several light intensities were compared with the distal pigment indices of crawfish on the white backgrounds. The results of both series of experiments were presented in figure 2.

In every instance that the state of the distal pigment of crawfish on a black background was compared at the same intensity of illumination with the state of the distal pigment of crawfish on a white background the average distal pigment index for the specimens on the black background was less than the index of crawfish on the white background.

White pans reflected 50% of the incident light. The black background reflected $\frac{1}{65}$ the light intensity a white background reflected. The average distal pigment indices for animals on a black background were therefore plotted in figure 2 not according to the logarithm of the illumination but instead as $\frac{1}{65}$ of this value. When the values for animals on a black background were plotted as the logarithm of $\frac{1}{65}$ of the incident light these average distal pigment indices fell on the curve obtained for animals on a white background. Because the indices obtained from the crawfish on both the black and white backgrounds fell on the same curve when this correction for reflected light was made the behavior of the retinal pigments could not have been due to an albedo response but must have been simply a response to the brightness of the visual field. There was no necessity to plot the indices for the crawfish on black and on white backgrounds in terms of the reflected light because a shift to the left of 50% on a logarithmic plot represents an insignificant shift. Also the relationship between the values obtained from the white and black backgrounds was the same whether incident or reflected light intensities were used.

A direct sigmoid relationship between intensity of illumination and average distal pigment index was evident. Distal retinal pigment of *Cambarellus* was functional over a wide range of intensities, from 0.00352 to 3500 ft. c. Between these intensities the crawfish exhibited adaptations of their retinal pigment that lay between dark adaptation and maximal light adaptation with distal pigment indices of 0.006 and 0.10 respectively.

*Rates of light and dark adaptation
of distal retinal pigment*

Five white enameled pans each containing 6 *Cambarellus* from the stock aquaria were placed in darkness for two hours when the average distal pigment index of the animals in one

pan was determined. The 4 remaining pans were then placed under an illumination of 40 ft. c. The average distal pigment index of 6 crawfish was then determined 5, 15, 30, and 60 minutes after the pans were put in light.

In a second series of experiments 5 pans containing 6 crawfish each were placed under an illumination of 40 ft. c. for two hours. At the end of this period the average distal pigment index of the crawfish in one pan was determined.

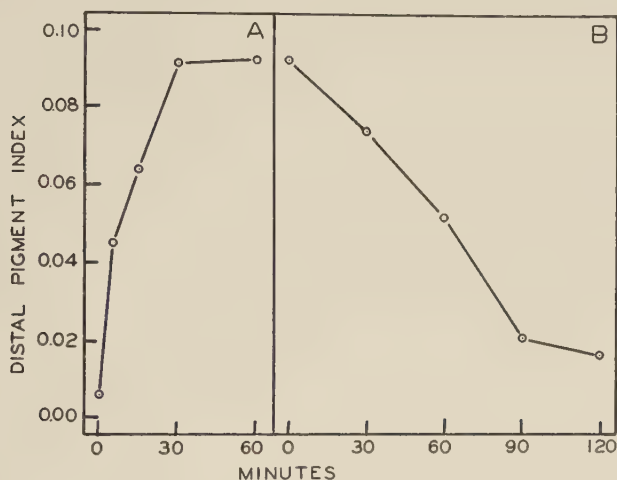


Fig. 3 Rate of light adaptation (A) and dark adaptation (B) of distal retinal pigment of *Cambarus* after transfer from darkness to light (A) and from light to darkness (B).

The 4 remaining pans were placed in darkness and the average distal pigment index of 6 crawfish was determined 30, 60, 90, and 120 minutes from the time the pans were placed in the darkroom. A different pan of crawfish was used for each determination.

Both experiments were repeated once. Data for the rates of light and dark adaptation are presented in figure 3. Each point represents the average of 12 crawfish. Light adaptation was completed in 30 minutes (fig. 3A); dark adaptation in 90 minutes (fig. 3B).

*Relationship between time in light and
rate of re-dark-adaptation*

Thirteen white enameled pans each containing 5 *Cambarellus* were placed in darkness for two hours to dark adapt the crawfish. The average distal pigment index of the crawfish in one pan was then determined. The remaining 12 pans were removed from the darkroom to an illumination of 40 ft. c. After 5 minutes in light three pans were returned to darkness, three more after 15 minutes in light and three more after 25 minutes in light. Whenever three pans were returned

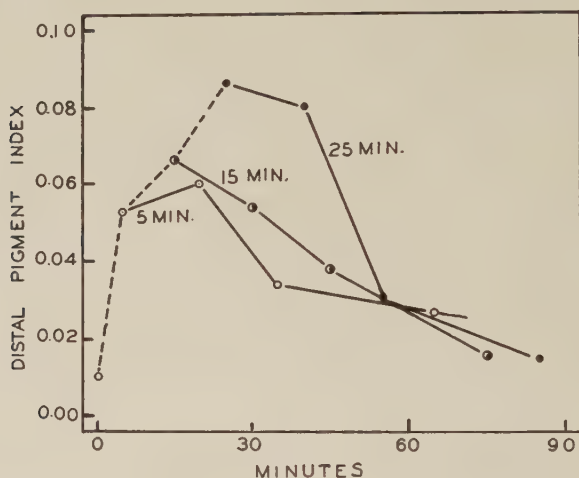


Fig. 4 Relationship between time under an illumination of 40 ft. c. and rate of re-dark-adaptation.

to darkness the average distal pigment index of the crawfish in a fourth pan was determined. The average distal pigment index of the *Cambarellus* in one pan of each of the three series was determined 15, 30, and 60 minutes following the return of each set of pans to darkness.

The experiment was repeated once and the results have been presented in figure 4 where each point represents the average of 10 crawfish. The dashed line connects the indices determined at the time each set of three pans was returned to darkness.

Inspection of the three curves with solid lines showed that the rate of re-dark-adaptation was a function of the time in light; the longer the time spent in light the greater was the rate of re-dark-adaptation.

Crawfish illuminated for 5 minutes continued their light adaptational process when returned to darkness before they began to dark adapt. However, the rate of light adaptation in darkness was less than the rate of light adaptation of crawfish kept in light. Crawfish that received 15 and 25 minutes of illumination did not show continued light adaptation upon being returned to darkness. The response of crawfish placed in light for 5 minutes suggested that a light adapting hormone was liberated into the blood during the 5 minutes of illumination and continued to function after the crawfish were returned to darkness. The increased rate of dark adaptation with increased time in light suggested that a large quantity of light adapting principle was liberated into the blood under the stimulus of a dark-to-light change and that the concentration in the blood rapidly decreased as the distal retinal pigment approached the fully light adapted condition. During the period in light the crawfish could also have been synthesizing a dark adapting hormone.

*Relationship between time in light and
rate of re-light-adaptation*

Sixteen white enameled pans, each containing 5 *Cambarellus*, were placed under an illumination of 40 ft. c. for two hours when the average distal pigment index of 5 crawfish in one pan was determined. The remaining pans were placed in darkness. After 20 minutes 4 of the pans were returned to the 40 ft. c. illumination, 4 more pans were returned to the illumination after 45 minutes in darkness and 4 more after 70 minutes in darkness. Each time a group of pans was placed at 40 ft. c. the average distal pigment index of 5 animals from one pan was determined and the crawfish discarded. The average distal pigment index of 5 crawfish was

determined 15, 30, 45, and 60 minutes after return to an illumination of 40 ft. c. The experiment was repeated once. The results have been presented in figure 5 where each point represents the average of 10 crawfish. The rate of re-light-adaptation was a direct function of the time spent in darkness. Apparently during the interval in darkness the crawfish recharged their sources of light adapting hormone. The quantity of available light adapting hormone must have decreased during the time the crawfish were maintained at 40 ft. c.

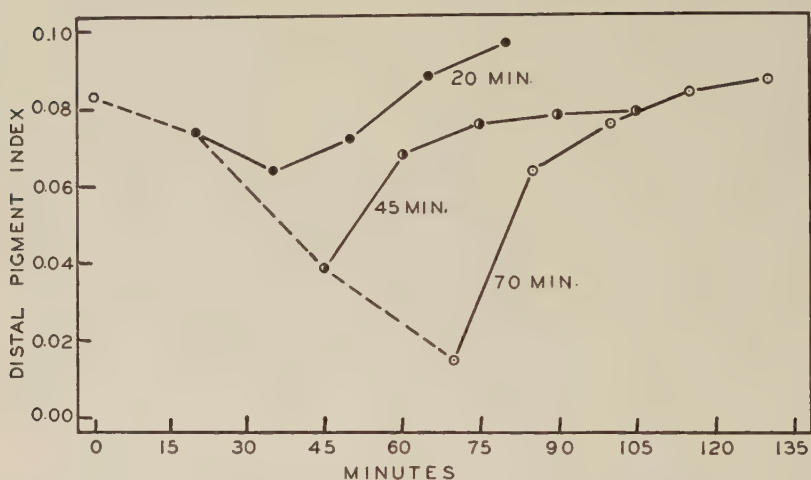


Fig. 5 Relationship between time in darkness and rate of re-light-adaptation under an illumination of 40 ft. c.

Crawfish in darkness for 20 minutes continued to dark adapt although at a diminished rate following the return to light. This continuation of the process of dark adaptation was probably due to secretion of a dark adapting hormone that continued to show its effect when the animals were in light until antagonized by a light adapting factor.

Response of distal retinal pigment to extracts of eyestalks and central nervous organs

The following experiments were devised to determine the sources of the blood-borne factors involved in adaptation of

the retinal pigments. For the first experiment 9 white enameled pans each containing 5 crawfish were placed in the darkroom for at least 4 hours to assure maximum dark adaptation of the distal retinal pigment. At the end of this period the average distal pigment index of the crawfish in one pan was determined. Each animal in 4 of the remaining pans received an injection of one eyestalk extracted in 0.02 ml of van Harreveld's solution. The crawfish in the 4 remaining pans received van Harreveld's solution as a control. The

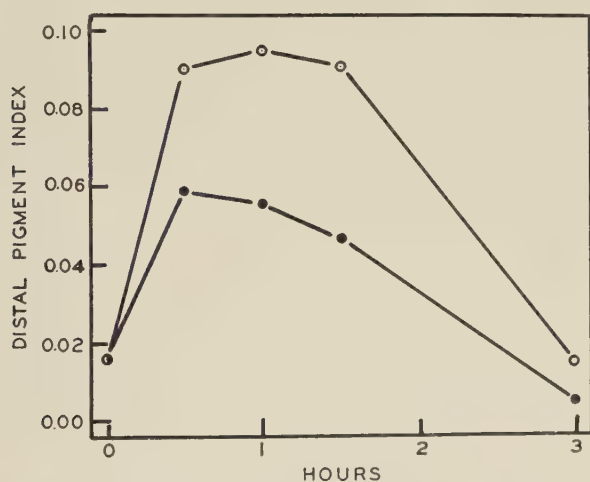


Fig. 6 Responses of dark adapted *Cambarellus* to eyestalk extract administered during a brief period of illumination. Circles, eyestalk extract; dots, van Harreveld's solution as a control.

animals were injected in the darkroom under a red photographic light known to produce a submaximal degree of light adaptation of the distal pigment. Thirty, 60, 90, and 180 minutes from the time of the injections one experimental and one control pan were removed from the darkroom and the average pigment index of the animals in each pan was determined. The experiment was repeated once. The results are presented in figure 6 where each point represents the average of 10 crawfish. The red photographic light caused a light adaptational response but the response of the crawfish that

received eyestalk extract was much greater, demonstrative of the presence of a light adapting factor in the eyestalk.

In another experiment 7 white enameled pans each containing 5 crawfish were placed at 40 ft. c. for two hours and then placed in darkness. After 10 minutes in darkness the animals in three pans were injected under the red photographic light with an extract containing $\frac{1}{2}$ of the supraesophageal ganglia of a crawfish extracted in 0.02 ml of van Harreveld's solution. The crawfish in three other pans were

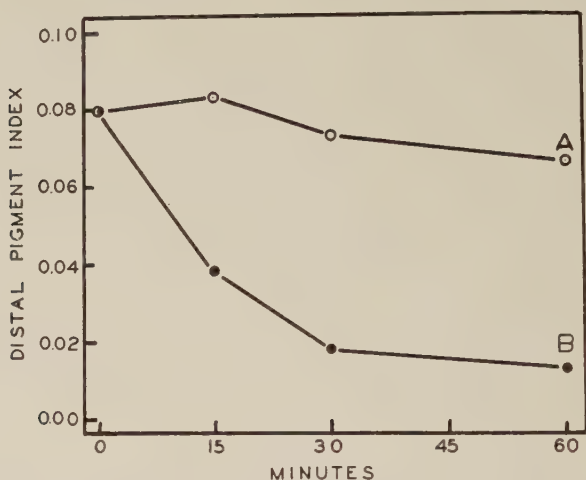


Fig. 7 Comparison of responses of *Cambarellus* to supraesophageal ganglia extract (A) and van Harreveld's solution (B) administered 10 minutes after the crawfish were put in darkness.

each injected with 0.02 ml of van Harreveld's solution as a control. The average distal pigment index of the 5 crawfish in the remaining pan was determined for the zero point. The average distal pigment indices of the crawfish in a control and an experimental pan were determined 15, 30, and 60 minutes from the time of the injections. The experiment was repeated once. The results are presented in figure 7 where each point represents the average of 10 crawfish. The distal retinal pigment of crawfish that received supraesophageal ganglia extract showed little tendency to dark adapt during

the hour following the injection. But the distal retinal pigment of the control group was completely dark adapted at the end of this period. The supraesophageal ganglia must also be a source of distal retinal pigment light adapting hormone.

DISCUSSION

The distal retinal pigment of *Cambarellus* was functional over a wider range of intensities than the pigment of *Palaemonetes vulgaris*. Between 50 and 4000 ft. c. the distal retinal pigment of *Palaemonetes* was maximally light adapted (Sandeén and Brown, '52). The distal pigment of *Cambarellus* did not become maximally light-adapted at intensities less than 3500 ft. c. The distal pigment of both species was in a more proximal position in specimens on a white background than on a black background at the same intensity of illumination. This response was not an albedo response but a response to decreased illumination caused by the black background.

In both *Palaemonetes* and *Cambarellus* the distal retinal pigment light adapting hormone was demonstrated in two ways. One method employed differences in rates of re-dark-adaptation following a period of illumination (fig. 4). Crawfish that were in light for 5 minutes continued to light adapt upon return to darkness, showing that a light adapting factor had been released into the blood and continued to act for a period of time in darkness. The experiment also suggested a buildup of dark adapting ability in light, probably a hormone, because the longer the crawfish were kept in light the faster were they able to re-dark-adapt. Extracts of eyestalks and central nervous organs contained a light adapting hormone (figs. 6 and 7).

Evidence in favor of a retinal pigment dark adapting hormone in *Cambarellus* could be drawn only from rates of re-light-adaptation (fig. 5). Crawfish that had been in darkness for 20 minutes continued to dark adapt when returned to light, showing that a dark adapting hormone must have been released into the circulation when crawfish were placed in darkness.

SUMMARY AND CONCLUSIONS

1. Distal retinal pigment of the dwarf crawfish, *Cambarellus shufeldti*, showed a graded response to intensity of illumination between 0.00352 and 3500 ft. c.

2. Distal retinal pigment was less light adapted when the *Cambarellus* were on a black background than on a white background at the same light intensity. The black background functioned to decrease the brightness of the visual field.

3. Light adaptation required 30 minutes; dark adaptation 90 minutes.

4. Light adaptation was due to secretion of light adapting hormone by eyestalks and central nervous organs.

5. The rate of light adaptation increased to a maximum after the crawfish had been in darkness for two hours. This increase was probably due to accumulation of light adapting hormone.

6. Evidence for a dark adapting hormone was based upon differences in rates of re-light-adaptation.

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TWENTY-FOUR HOUR RHYTHM OF DISTAL RETINAL PIGMENT MIGRATION IN THE DWARF CRAWFISH ¹

MILTON FINGERMAN AND MILDRED E. LOWE
*Department of Zoology, Newcomb College, Tulane University,
New Orleans 18, Louisiana*

FOUR FIGURES

Persistent 24-hour rhythms of migration of retinal pigments in the compound eye have been reported for several crustaceans. Review of the literature disclosed that any one or any combination of the three pigments, distal, proximal, and reflecting, may exhibit a 24-hour rhythmicity (Brown, '44; Kleinholz, '49). These rhythms may persist in darkness only, in light alone or under either condition.

Proximal retinal pigment of the crawfish *Orconectes virilis* exhibited in darkness a persistent 24-hour rhythm of migration from the light adapted to the dark adapted state (Bennitt, '32). In another crawfish, *Cambarus bartoni*, both the proximal and distal retinal pigments have a 24-hour rhythm of migration (Welsh, '41). Webb and Brown ('53) demonstrated a rhythm of the distal retinal pigment in the prawn *Palaemonetes vulgaris*. This rhythm expressed itself only when the illumination was of such an intensity that the distal retinal pigment was in an intermediate state of light adaptation.

The rhythmical mechanism controlling retinal pigment migration is similar to the physiological basis for rhythms of migration of chromatophore pigments. Welsh ('41) demonstrated that the frequency of the daily rhythm of retinal

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pigment migration in *Cambarus bartoni* was independent of temperature between 7° and 21°C. Brown and Webb ('48) demonstrated the same property for the chromatophore pigmentary rhythm in the fiddler crab *Uca pugilator* between 6° and 26°C. Smith ('48) found a retinal pigment rhythm in the grapsoid crabs, *Hemigrapsus oregonensis*, *H. nudus* and *Pachygrapsus crassipes*, in darkness only. If the crabs were placed in darkness at 7 A.M., their rhythms were displaced 6 hours with respect to solar day-night. Brown and Webb ('49) found a similar 6 hour shift occurred in the melanophore pigmentary rhythm of *Uca* placed in darkness at 7 A.M.

The rate of oxygen consumption of the dwarf crawfish, *Cambarellus shufeldti*, was continuously recorded for 24-hour periods (Fingerman, '55). Analysis of the data revealed a 24-hour rhythm of metabolic rate.

Endocrine regulation of the distal retinal pigment of *Cambarellus* has been investigated (Fingerman, '56, '57). Light-adaptation was due to secretion of light adapting hormone by eyestalks and central nervous organs. Dark adaptation was probably due to dark adapting hormone from the same organs. The present investigation was undertaken to determine if a 24-hour rhythm controls at least in part the state of the distal retinal pigment of the dwarf crawfish.

MATERIALS AND METHODS

Specimens of the dwarf crawfish, *Cambarellus shufeldti*, collected at frequent intervals at Crown Point, Louisiana, were used in the experiments. Crawfish in the stock aquaria were kept under normal day-night conditions to prevent impairment of the ability to light adapt fully (Fingerman, '56).

The state of the distal retinal pigment was expressed as the distal pigment index. The method of determining this index for *Cambarellus* has been described fully (Fingerman, '57). In brief, the index was determined by measuring with the aid of a stereoscopic dissecting microscope and an ocular micrometer the width of the transparent distal portion of the eye (A) and the total length of the eye along a line parallel to the

long axis of the eyestalk (B). The total length was measured from the distal edge of the cornea to the apex of the notch at the proximal portion of the eye on the dorsal surface of the eyestalk. The ratio of the two measurements (A/B) constituted the distal pigment index. The width of the clear area increased as the eye became more light-adapted. Crawfish were used without regard to size or sex. Use of a ratio in the form of the distal pigment index minimized effects due to size differences. For each experiment crawfish were placed in white enameled pans with a bottom diameter of 14.5 cm that contained aerated tap water one inch deep.

EXPERIMENTS AND RESULTS

Influence of time of day on rate of dark adaptation

Fifteen normal crawfish taken from the stock aquaria were placed into each of four white enameled pans at 8 A.M. The pans were then placed under an illumination of 40 ft. c. Beginning at 9:30 A.M. and every two hours thereafter for 30 hours one pan was placed in darkness for 30 minutes. At the end of this period in the darkroom the average distal pigment index of 10 of the crawfish in the pan was determined. This pan of crawfish was then returned to the 40 ft. c. illumination. The four pans were used in sequence so that each pan of crawfish was used only once every eight hours.

A 24-hour rhythm was evidenced by the degree of dark adaptation of the distal retinal pigment that had occurred during the 30 minutes in darkness (fig. 1). The pigment was the least light adapted after 30 minutes in darkness at midnight and most light adapted after 30 minutes in darkness at noon. The rhythm was symmetrical about noon and midnight. At noon in all probability more light adapting hormone was in the blood than at midnight, accounting for the difference in rate of dark adaptation.

Influence of time of day upon response to light

At 10 A.M. 15 *Cambarellus* from the stock aquaria were placed into each of four white enameled pans. At noon one

of the pans was carried into the darkroom. At 1:55 P.M. the pan was taken from the darkroom and placed under an illumination of 40 ft. c. for five minutes. The pan was then returned to darkness with the second of the four pans. At 2:30 P.M.

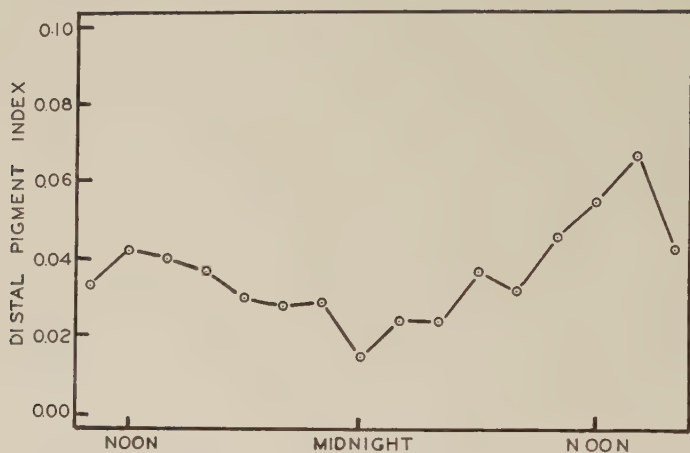


Fig. 1 Average distal pigment index of *Cambarellus* 30 minutes after being placed in darkness at different times of day.

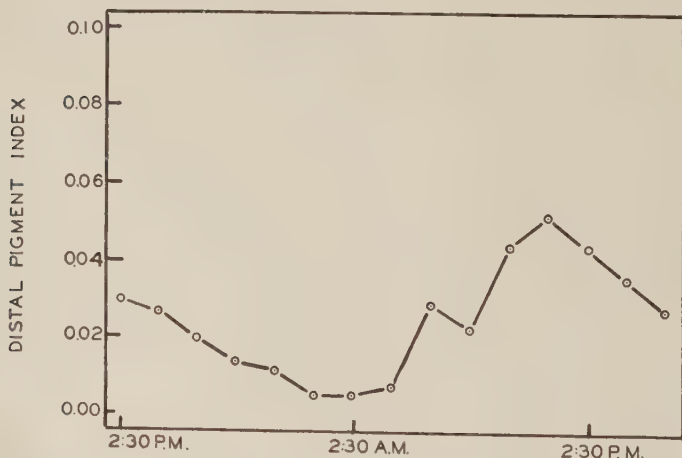


Fig. 2 Average distal pigment index of previously dark adapted *Cambarellus* 30 minutes after receiving a five minute 40 ft. c. light exposure at different times of day. The crawfish were returned to darkness after the light exposure.

the average distal pigment index of 10 crawfish in the first pan was determined. The crawfish placed in the darkroom at 2 P.M. were given a similar light exposure at 3:55 P.M. At 4:30 P.M. the average distal pigment index of 10 crawfish in the pan was determined. The procedure was repeated every two hours until 6:30 P.M. of the following day. These four pans were also used in sequence.

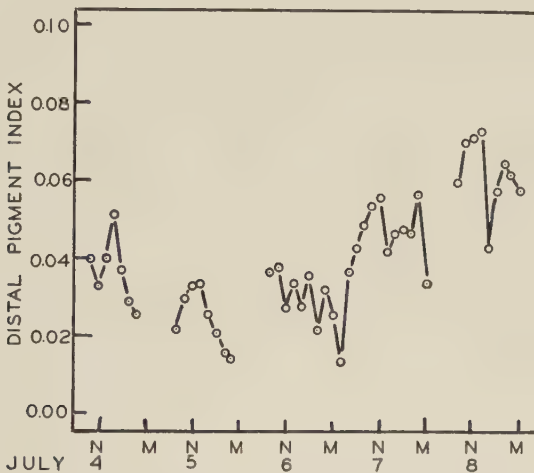


Fig. 3 Twenty-four hour rhythm of *Cambarellus* unable to light-adapt fully at the start of the observations. The crawfish were maintained on a white background under an illumination of 40 ft. c. for five days.

The data were used to prepare figure 2. The character of the 24-hour rhythm determined by this method was the same as the rhythm determined previously (fig. 1). Both rhythms were symmetrical about noon and midnight with the largest distal pigment indices having occurred at noon.

Twenty-four hour rhythm of Cambarellus with limited ability to light adapt

A white enameled pan containing 15 *Cambarellus* with impaired ability to light adapt because they had been kept overnight at an illumination of 0.025 ft. c. was placed under an

following day, July 6 through July 13, 1955. The times when observations were made are shown in figure 4.

Normal crawfish also showed a 24-hour rhythm symmetrical about noon and midnight. The amplitude of the rhythm of normal crawfish decreased with time. The distal retinal pigment remained fully light adapted for a greater number of hours during each subsequent 24-hour period. On July 12 and 13 the rhythm was almost completely inhibited by the constant illumination. Very little fluctuation of the state of the pigment was evident.

DISCUSSION

The results described above have demonstrated the daily rhythm of migration of the distal retinal pigment of *Cambararellus* in three ways: (1) rate of dark adaptation throughout the 24-hour day, (2) response to a light exposure throughout the day, and (3) direct observation throughout the day of the degree of light adaptation of the distal retinal pigment of crawfish kept under constant illumination. The character of the rhythm determined from each type of experiment was identical. The maximum of the 24-hour curve occurred at noon and the minimum at midnight. The character of the 24-hour rhythm of metabolic rate in *Cambararellus* was different from the distal retinal pigment rhythm. The former was bimodal with maxima in the morning and afternoon and minima at noon and midnight (Fingerman, '55).

The retinal pigment rhythm was probably due to fluctuations in (1) titer of light adapting hormone in the blood and (2) ability of the crawfish to synthesize and secrete light adapting hormone. At noon the crawfish had a large quantity of light adapting hormone in the blood and in the glands that produce the hormone. At midnight the concentrations were low. In contrast, the retinal pigment rhythm of *Palaemonetes* was interpreted as due to differences in the quantity of dark adapting hormone available (Webb and Brown, '53).

Inhibition of the distal retinal pigment rhythm after a week in constant light of 40 ft. c. on a white background was similar

to inhibition of the 24-hour chromatophore rhythm of specimens of *Uca pugnax* also kept under a constant illumination of 40 ft. c. on a white background (Brown and Hines, '52). Amplitudes of the rhythms of both *Cambarellus* and *Uca* gradually decreased with time. The distal retinal pigment tended to remain completely light adapted throughout the 24-hour day after six days under constant illumination (fig. 4). Normally *Uca* is dark by day and light by night. The pigment in the melanophores of *Uca* tended to remain completely dispersed throughout the 24-hour day under constant illumination on a white background. As further information concerning the nature of retinal pigment rhythms is obtained, the similarity between retinal pigment and chromatophore rhythmical mechanisms becomes more striking.

SUMMARY AND CONCLUSIONS

1. Distal retinal pigment of the dwarf crawfish, *Cambarellus shufeldti*, showed a 24-hour rhythm of migration. Differences in (1) rate of dark adaptation throughout the 24-hour day and (2) ability of dark adapted *Cambarellus* to respond to a light exposure were evident. Direct observation of the distal retinal pigment of crawfish exposed to constant illumination throughout the day also revealed the 24-hour rhythm.

2. Distal retinal pigment of crawfish kept under an illumination of 40 ft. c. intensity was most light adapted at noon and least light adapted at midnight. Dark adaptation was most rapid at midnight and slowest at noon. Maximal response to a light exposure occurred at noon and minimal response occurred at midnight.

3. The rhythm was interpreted as due to changes throughout the day of the quantity of light adapting hormone in the blood and in the glands available for immediate secretion in response to a light exposure.

4. Similarities between the controlling mechanisms of 24-hour retinal pigment rhythms and chromatophore rhythms are discussed.

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THE LOCALIZED CHARACTER OF ULTRAVIOLET EFFECTS ON THE URODELE FORELIMB ¹

E. G. BUTLER, H. F. BLUM AND S. E. SCHMIDT

*Department of Biology, Princeton University, Princeton, New Jersey and the
National Cancer Institute, Bethesda, Maryland ²*

FIVE FIGURES

In a recent paper (Blum, et al., '57) were presented the results of exposing the forearm of *Amblystoma* larvae to ultraviolet radiation. In particular, data were obtained on the regression of the irradiated forearm, the retardation in regeneration which resulted when amputation was made through the irradiated region, and the abnormalities which occurred in the regenerates.

It has seemed desirable to supplement the previous observations by determining whether changes produced by ultraviolet light have any effect on the regenerative capacity of an unirradiated region which is in close physiological association with an irradiated region through circulatory and nervous pathways. Specifically, the primary purpose of the present investigation has been to study the question, whether irradiation of the proximal segment of the limb (the upper arm) of a urodele larva will have an effect on the regenerative capacity or will alter the growth pattern of the unirradiated distal segments of the limb (the forearm and hand).

MATERIALS AND METHODS

The methods of irradiation, illumination, and measurement of limb growth have been described previously in this journal

¹ This investigation was supported in part by a research grant, C-1499, from the National Cancer Institute, of the National Institutes of Health, Public Health Service.

² National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

(Blum, et al., '57). In the present experiments, 208 larvae of *Amblystoma opacum* reared in the laboratory from eggs collected in North Carolina, were selected as described in the preceding paper. The average dimensions at the beginning of the experiment were as follows: over-all length of larvae, snout to tail tip, 23.5 mm (S. D. .81 mm); upper arm length, 1.62 mm (S. D. 0.10 mm); forearm length, 1.74 mm (S. D. 0.12 mm).

The right upper arm (see figure 1) of all animals was exposed to 6.2×10^7 ergs per cm^2 of wavelengths 0.313μ and shorter. Beginning immediately after irradiation with ultra-

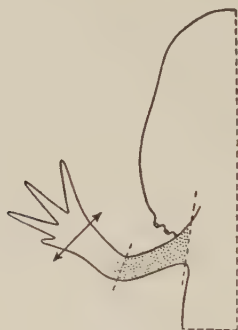


Figure 1

violet light, half of the animals were illuminated with visible light for two days under the conditions previously described; the other half of the animals were kept in darkness during this period. After two days all the animals were kept in the dark, except for the short periods required for observations and measurements. All animals were maintained in constant temperature chambers at 20°C at all times after irradiation.

Amputation of both right and left forearms at the distal end of the radius and ulna (as indicated in figure 1) was carried out in groups of 52 animals each. Half of the animals within each amputation group had been illuminated subsequent to ultraviolet irradiation, half had been kept continuously in the dark. On the basis of the time of amputation, there were four

different groups: in one group, amputation was made three days post-irradiation; in a second group, 10 days post-irradiation; in a third group 17 days post-irradiation; and, in a fourth group, 25 days post-irradiation.

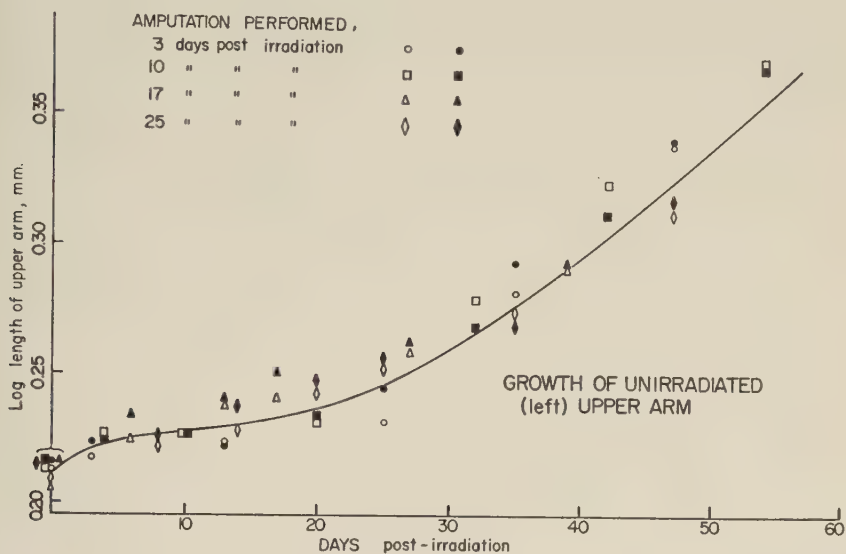


Figure 2

EXPERIMENTAL RESULTS

Rate of growth

Measurements of the lengths of both right (irradiated) and left (unirradiated) upper arms were made as follows: just previous to the time of irradiation; just before amputation; and at such other times as are indicated in figures 2 and 4. Up to the time of amputation, corresponding measurements were made of the forearms, as shown in figures 3 and 5. The growth of the control left limb is represented in figures 2 (upper arm) and 3 (forearm). The growth of the right limb is shown in figures 4 (irradiated upper arm) and 5 (unirradiated forearm); in these figures the average growth curve of the controls is reproduced as a broken line for comparison. Somewhat greater variability is indicated in these experiments than was found for those on *A. punctatum*.

previously described (Blum, et al., '57). This may be associated with greater genetic variability of the material, since the eggs of *A. opacum* are deposited singly, whereas in *A. punctatum* all the eggs of a single clutch (ordinarily 100 or more) are held together in a gelatinous mass, thus assuring that large numbers of eggs come from the same parents.

It is clearly shown in figures 2 and 3, as in all of our previous experiments, that there is no influence of illumination

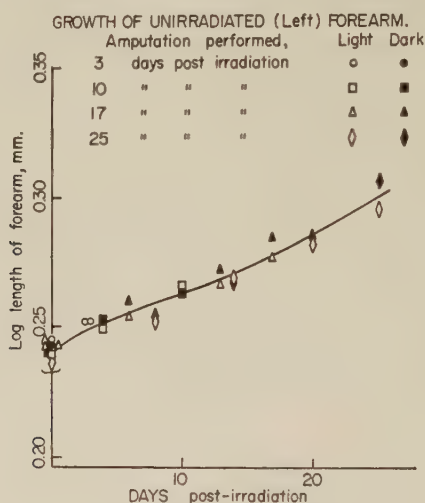


Figure 3

on the growth of unirradiated control limbs. It is clear from figure 4 that ultraviolet radiation brings about a decrease in the growth rate of the upper arm, and that illumination for only a short period (2 days) immediately following exposure to ultraviolet light ameliorates the effect of the latter. The same kind of effect is clearly indicated in figure 5 for the growth of the shielded forearm on the irradiated right side; this latter result undoubtedly reflects the unavoidable error in delimiting the area of irradiation (see figure 1), the proximal end of the forearm always receiving an uncertain amount of ultraviolet light. This situation corresponds to the effect

on the upper arm when the forearm was irradiated, and to the restricted irradiation of the elbow region, as described in previous papers (Butler, et al., '55; Blum, et al., '57).

Certain differences are to be noted between the effects on the growth of the upper arm and on the growth of the forearm, as indicated in figures 4 and 5. In the case of the upper arm, irradiation is followed by an immediate negative growth, indi-

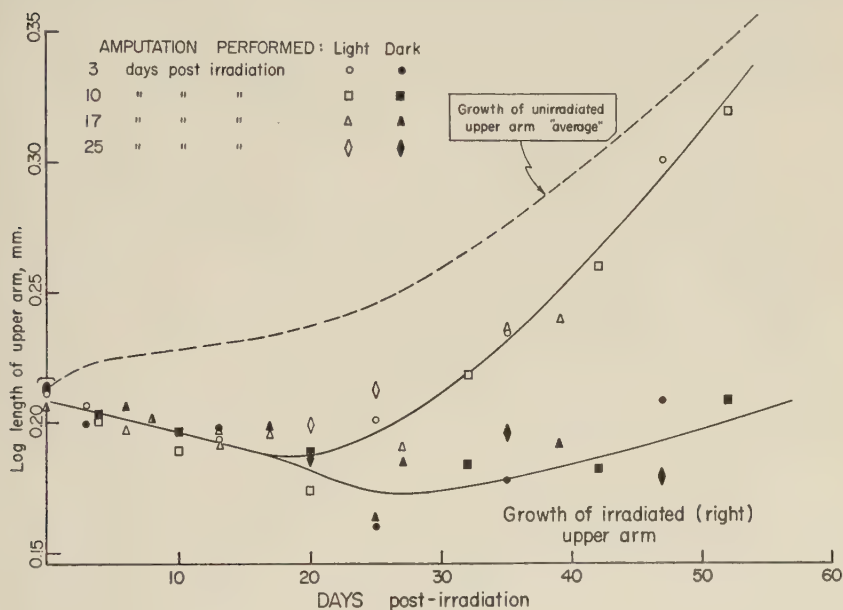


Figure 4

cating that regression (presumably involving the removal of material) is proceeding faster than cell proliferation. About 20 days post-irradiation, growth becomes positive for the "light animals," and somewhat later for the "dark animals." In the shielded forearms of the irradiated right limb (figure 5), on the contrary, growth continues for about ten days at the same positive rate as that shown by the controls. After this time the "light animals" show a lowered positive growth rate and the "dark animals" a negative one. These results resemble those found in the case of irradiated forearms in the

previous experiments (Blum, et al., '57). It is especially noteworthy, that in all our experiments we find that the effects on growth rate may take different shapes according to the regions irradiated, although the general direction is always the same. Dose of radiation may also be a factor. Although the dose per unit area was the same for the upper arm in the present case, as for the forearm in the previous experiments, the effective dose may have been considerably greater because

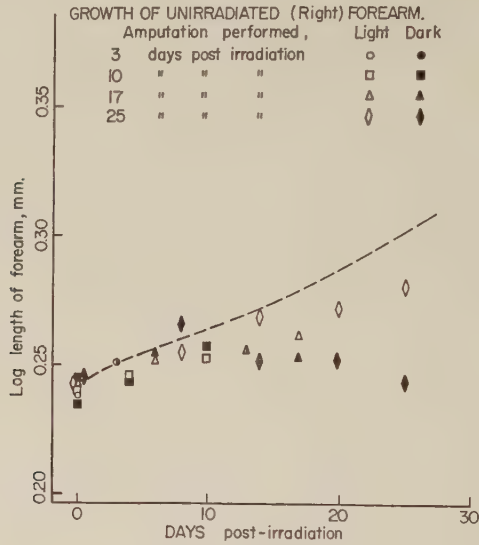


Figure 5

the forearm is thinner and hence the relative penetration is greater. These differences indicate the need for care in the interpretation of such experiments because of anatomical factors concerned, the influence of which it is often difficult to evaluate.

Regenerative capacity

Amputation at the distal end of the forearm was carried out in all larvae as described under Materials and Methods. After amputation the animals were examined frequently with respect to the relative rates and the normality of regeneration of the

forelimbs. Records were made specifically on the extent and character of the regeneration on the 10th, the 22nd, the 32nd and the 44th day post-amputation.

At no time during the course of the observations was there evidence of any significant difference in the rate of regeneration of the right limbs, which had been irradiated above the level of amputation, as compared with the totally unirradiated left limbs. This was true regardless of whether the animals had been illuminated subsequent to irradiation, and also regardless of the time which had elapsed between irradiation and amputation. Extensive data were accumulated which

TABLE 1

Extent of regeneration twenty-two days after amputation
All amputations through distal end of forearm (see fig. 1)

| STAGE OF REGENERATE | UPPER ARM IRRADIATED (186 cases) | | NO IRRADIATION (186 cases) | |
|------------------------|-------------------------------------|---------------------------------|-------------------------------|--------------------|
| | Animals in light | Animals in dark ¹ | Animals in light | Animals in dark |
| 2 digits | 11% | 19% | 10% | 13% |
| 3 digits | 71% | 61% | 65% | 62% |
| 4 digits | 18% | 18% | 25% | 25% |

¹ Two abnormal regenerates are not included in this table.

substantiate these statements. As an example, table 1 presents a sample of the data taken from the records 22 days after amputation. No essential differences appeared in the records at 10, 32 and 44 days post-amputation. Only four abnormalities appeared among all of the regenerates concerned, 3 in irradiated limbs and one in a control limb.³

From the present experiments it seems clear that the effects which ultraviolet is known to exert on regenerative growth (see Butler, et al., '55; Blum, et al., '57) are limited specifically to the region irradiated. On the basis of present evidence, the regenerative capacity of a region adjacent to an irradiated region remains unaffected, regardless of the close physiologi-

³ Two of the abnormalities on irradiated limbs were supernumerary digits at the wrist level. These undoubtedly were the result of inaccurate shielding of the limb at the time of irradiation.

cal association by way of nerves, blood vessels and other structures. So long as the level of amputation is outside of the irradiated area, even though it be distal to it, a normal regenerate will be established. These results are remindful of those previously obtained through the use of x-irradiation (Butler and O'Brien, '42), and again emphasize the local nature of regenerative activity in the urodele limb.

SUMMARY

1. Localized ultraviolet irradiation of the upper arm of larval *Amblystoma opacum* results in changes in the growth pattern of this segment of the limb. These changes are ameliorated by illumination with visible light for a period immediately following the ultraviolet.

2. Irradiation of the upper arm brings about some changes in the growth pattern of the unirradiated forearm of the same limb. These are undoubtedly the result of our inability completely to shield the proximal end of the forearm from the radiation.

3. Irradiation of the upper arm does not affect the regenerative capacity of the unirradiated forearm of the same limb, when amputation is distal to the irradiated region. Regeneration of such a forearm is as rapid and complete as regeneration of the forearm of the totally unirradiated contralateral limb. This is in contrast to the results of amputation of an irradiated forelimb, as previously reported.

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NUTRITION AND METABOLISM OF MARINE BACTERIA. III. THE RELATION OF SODIUM AND POTASSIUM TO GROWTH

ROBERT A. MACLEOD AND E. ONOFREY

The Fisheries Research Board of Canada, Technological Station, Vancouver, B. C.

INTRODUCTION

A number of marine bacteria have been found to require both Na^+ and K^+ for growth (MacLeod et al., '54, '56). Although many bacteria have been shown to require K^+ (Haynes et al., '54) the need for Na^+ by these forms appears to be almost unique among bacteria so far investigated. In the case of only one representative of any other group of bacteria has evidence for a Na^+ requirement been obtained. The halophile *Pseudomonas salinaria* has recently been shown to require Na^+ for growth (Brown and Gibbons, '55).

In this study the quantitative requirements for Na^+ and K^+ and the specificity of these requirements have been determined for three marine bacteria. Information regarding the intracellular concentrations of the ions relative to their concentration in the medium has been obtained. The findings indicate that there is a marked similarity between marine bacterial cells and animal cells as regards both the relationship between intracellular and extracellular Na^+ and K^+ levels and the quantitative requirements of both types of cells for these ions for growth.

MATERIAL AND METHODS

Cultures. The sources of the organisms and the general methods used in their study have been described (MacLeod et al., '54, '56). Three of the organisms studied previously were

selected for this work. They have been tentatively identified as B₉, a *Flavobacterium*; B₁₀, a *Corynebacterium*, and B₁₆, a *Mycoplana*.¹

Assay procedures. The composition of the basal and inoculum media, the preparation of the inocula, and the assay procedures used have been described (MacLeod and Onofrey, '56).

Analytical methods. Analyses for Na⁺ and K⁺ were made with a flame photometer attachment for a Beckman DU spectrophotometer equipped with a photomultiplier. Na⁺ and K⁺ standards were prepared containing ions at levels similar to those present in the solutions to be analyzed. Analyses were carried out directly on the untreated media and on dry-ashed cell preparations.

RESULTS

Response to Na⁺

The bacteria investigated would not grow in a medium prepared with artificial sea water if Na⁺ was omitted (MacLeod and Onofrey, '56). Growth could not be restored if Na⁺ was replaced by equimolar concentrations of either K⁺ or glycerol. This suggested a specific function for Na⁺ in the growth of the organisms apart from any role it might play in maintaining proper osmotic pressure relations for the cells.

The response of the organisms to various levels of Na⁺ in a Na⁺-deficient medium was determined. The results obtained with organism B₁₆ are presented in table 1. It is evident that the level of Na⁺ in the medium affected both the rate and extent of growth. Both the maximum rate and extent of growth was achieved with 2 millimoles of Na⁺ per 10 ml of medium. This level of Na⁺ is slightly less than half that found in sea water. Below the 2 millimole level both the rate and the extent of growth were roughly proportional to the amount of Na⁺ added.

¹ A recent study of the classification of these organisms conducted by Dr. Einar Leifson has shown that B₁₀ is a species of the genus *Pseudomonas* and B₁₆ belongs to either the genus *Pseudomonas* or *Spirillum*. We are deeply indebted to Dr. Leifson for undertaking this investigation.

Growth occurred at the 0.3 millimole level of Na⁺ after a 10-day incubation period though not at still lower levels by 21 days. Once cultures had ceased growing they began to autolyze rapidly. Though not shown here, separate experiments have revealed that a level of 8 millimole of Na⁺ per 10 ml of medium inhibited the growth of all three organisms. This amount of Na⁺ is supplied in a medium containing approximately 5 per cent NaCl. Organisms B₉ and B₁₀ responded like B₁₆ to Na⁺ except that in the case of B₉ there was almost no tendency for the cultures to autolyze after growth had ceased.

TABLE 1

The response of marine bacterium B-16 to Na⁺ in a Na⁺ deficient medium

| Na ⁺ ¹ | INCUBATION TIME (HR.) | | | |
|---|-----------------------|-----|-----|-----|
| Millimoles/10 ml | 48 | 72 | 120 | 312 |
| Percent incident light transmitted ² | | | | |
| 0.0 | 100 | 100 | 100 | 100 |
| 0.1 | 100 | 100 | 100 | 100 |
| 0.3 | 100 | 100 | 100 | 44 |
| 0.5 | 100 | 100 | 89 | — |
| 0.7 | 98 | 39 | 35 | — |
| 1.0 | 86 | 39 | 46 | — |
| 1.3 | 34 | 30 | 60 | — |
| 2.0 | 26 | 30 | 52 | — |
| 3.0 | 25 | 34 | 51 | — |

¹ Added as NaCl.

² Evelyn colorimeter readings 660 mμ filter. Uninoculated medium = 100.

Since the level of Na⁺ required for optimum growth is considerably higher than that of other inorganic ions involved in bacterial nutrition (MacLeod and Snell, '47), it seemed not unlikely that at least part of the function of Na⁺ might be to maintain an appropriate osmotic pressure in the medium. To test this possibility, the response of the three organisms to Na⁺ was determined in the presence and absence of sucrose, since sucrose has a low rate of penetration into cells, at least in some biological systems (Collander and Börland, '33) and is not metabolized by cell suspensions of organism B₁₆. It was found that all of the organisms grew at slightly lower

levels of Na^+ in the presence of sucrose than in its absence, the sparing action being more pronounced after short incubation periods. It seemed evident from the results, however, that the maintenance of a high osmotic pressure in the medium was by no means the primary function of Na^+ in the growth of the cells.

Specificity of the Na^+ requirement

The ability of Li^+ , K^+ , Rb^+ and Cs^+ to replace or spare the requirement of the three organisms for Na^+ was determined.

TABLE 2

Response of marine bacterium B-10 to Na^+ in the presence and absence of K^+ added in excess of growth requirements

| Na ⁺ | INCUBATION TIME (HR.) | | | |
|-------------------------|---|-----|-----|-----|
| | 68 | | 241 | |
| <i>Millimoles/10 ml</i> | | | | |
| | K ⁺ added (<i>millimoles/10 ml</i>) | | | |
| | 0 | 1 | 0 | 1 |
| | Percent incident light transmitted ¹ | | | |
| 0.0 | 100 | 100 | 100 | 100 |
| 0.1 | 100 | 100 | 100 | 100 |
| 0.3 | 100 | 100 | 85 | 67 |
| 0.5 | 100 | 70 | 50 | 73 |
| 1.0 | 99 | 43 | 79 | 69 |
| 1.3 | 46 | 43 | 78 | 73 |
| 1.5 | 37 | 15 | 71 | 42 |
| 2.0 | 20 | 14 | 67 | 32 |

¹ See table 1.

None of the ions permitted growth when added in place of Na^+ to the deficient medium. When the response of each organism to Na^+ in the presence and absence of 1 millimole of each of the related ions was compared Li^+ , K^+ , and to a lesser extent Rb^+ had sparing actions for all three organisms after short incubation periods which were barely detectable after longer ones. Cs^+ , on the other hand, was somewhat inhibitory at these concentrations. The nature of the sparing actions obtained is illustrated in table 2 where the effect of K^+ on the

response of B₁₀ to Na⁺ is shown. It is evident that after 68 hr of incubation between 1.0 and 1.3 millimoles of Na⁺ per 10 ml was required for growth in the absence of K⁺ and between 0.3 and 0.5 millimoles in its presence. After 241 hr there was little difference in the Na⁺ requirement in the presence and absence of K⁺. This sparing action of K⁺ on the Na⁺ requirement was quite similar to that obtained with sucrose. The decrease in turbidity of some of the cultures on longer incubation was due to autolysis occurring after growth had ceased.

The possibility that the inhibitory action of Cs⁺ might be due to an ability of the ion to interfere with the utilization of Na⁺ was considered. No evidence of an antagonism between Na⁺ and Cs⁺ could be demonstrated for any of the organisms.

Response to K⁺

When the quantitative requirements of the organisms for K⁺ were determined it was found that only about one one-hundredth as much K⁺ as Na⁺ was needed. As in the case of Na⁺, the amount of K⁺ in the medium affected not only the extent but also the rate of growth of the organisms. These observations are illustrated in the response of organism B₁₀ to K⁺ after three periods of incubation. (Table 3, response in the absence of Cs⁺). It is evident that although maximum growth of the organism was obtained in 48 hr in the presence of sufficient K⁺, extensive additional growth occurred at suboptimal levels on longer incubation. Organisms B₉ and B₁₆ responded to K⁺ in the same way as B₁₀.

Neither Li⁺ nor Cs⁺ were able to replace K⁺ for the growth of the organisms. When Rb⁺ was tested it was found to be capable of replacing K⁺ but only when added in high concentrations. When the Rb⁺ salt used was analyzed with the aid of a flame spectrophotometer, it was found that the response to Rb⁺ could be accounted for quantitatively by the amount of K⁺ present as a contaminant. This was confirmed by removing all but traces of K⁺ from the Rb⁺ salt by ion exchange chromatography using Amberlite IR-120 and a pro-

cedure similar to that described by Cohn and Kohn '48). The response to the purified Rb^+ salt was again proportional to the K^+ present.

Cs^+ and Li^+ failed to replace K^+ and also did not promote growth in the presence of suboptimal levels of the ion. The apparent sparing action of Rb^+ could be accounted for again by the level of K^+ present as a contaminant in the Rb^+ salt.

TABLE 3

Response of marine bacterium B-10 to K^+ in the presence and absence of Cs^+

| K ⁺ | Cs ⁺ (millimoles/10 ml) | | | | | | | | |
|------------------|---|-----|-----|-----|-----|-----|-----|-----|-----|
| | 0.0 | | | 0.3 | | | 0.5 | | |
| Micromoles/10 ml | Time (hr.) | | | | | | | | |
| | 48 | 96 | 144 | 48 | 96 | 144 | 48 | 96 | 144 |
| | Percent incident light transmitted ¹ | | | | | | | | |
| 0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 1 | — | — | 100 | — | — | — | — | — | — |
| 1.5 | 100 | 100 | 98 | — | — | — | — | — | — |
| 2 | 98 | 97 | 58 | — | — | — | — | — | — |
| 3 | 72 | 59 | 71 | — | — | 100 | — | — | — |
| 4 | 38 | 77 | 87 | — | 100 | 89 | — | — | 100 |
| 10 | 9 | 28 | 37 | — | 88 | 15 | — | — | 98 |
| 30 | 5 | 19 | 26 | — | 4 | 9 | — | 100 | 7 |
| 100 | 6 | 21 | 29 | 100 | 5 | 16 | — | 19 | 20 |
| 1000 | 14 | 36 | 45 | 12 | 27 | 33 | 100 | 17 | 18 |

¹ See table 1.

Ion antagonism

When Li^+ , Rb^+ and Cs^+ were tested to determine their toxicity for the three organisms, Cs^+ was the only ion significantly inhibitory below a level of 2 millimoles per flask, and then only for organisms B_9 and B_{10} . Evidence showed that at least a part of the toxicity of Cs^+ was due to its ability to interfere with the response of the organisms to K^+ . This is evident from the results in table 3 where it can be seen that considerably more K^+ was required to obtain growth in 48 hr in the presence of 0.3 millimoles of Cs^+ than in its absence. At the higher Cs^+ level tested no growth was apparent in 48 hr even at the

highest level of K⁺ tested though growth did occur on longer incubation. It would thus appear that Cs⁺ interfered with the ability of K⁺ to affect both the rate and the extent of growth of the organism.

Ion uptake by growing cultures

Because of the size and specificity of the Na⁺ requirement, further information concerning the relation of the ion to the growth of the cells was sought. Cultures were grown in media containing suboptimal levels of Na⁺ until the amount

TABLE 4

Uptake of Na⁺ and K⁺ from the medium by cells of marine bacterium B-10 during growth

| TURBID- ITY ¹ | Na ⁺ in medium | | UPTAKE | TURBID- ITY ¹ | K ⁺ in medium | | UPTAKE |
|-----------------------------|------------------------------|--|--------|-----------------------------|------------------------------|-----------------------------|--------|
| | Before growth mg/10 ml | After growth ² mg/10 ml | | | Before growth μg/10 ml | After growth μg/10 ml | |
| 62 | 5.7 | 5.6 | + .1 | 96 | 118 | 113 | + 5 |
| 57 | 9.0 | 9.1 | — .1 | 91 | 141 | 131 | + 10 |
| 50 | 11.9 | 12.0 | — .1 | 75 | 136 | 121 | + 15 |
| 4 | 23.7 | 23.5 | + .2 | 42 | 162 | 139 | + 23 |
| | | | | 12 | 300 | 182 | + 118 |

¹ Percent of incident light transmitted.

² Cultures were incubated until growth ceased.

of growth permitted by the level of Na⁺ present was attained. The cells were removed by centrifugation and the Na⁺ concentration in the supernatant medium compared with that in an uninoculated control tube. The results, table 4, indicate that no net uptake of Na⁺ by the cells could be demonstrated by this method. When the same technique was applied to cultures grown in the presence of suboptimal levels of K⁺, evidence of uptake of K⁺ by the cells was obtained, though again considerable quantities of the ion remained in the medium after growth had ceased, (table 4). These results indicate that when growth is limited by the level of either Na⁺ or K⁺ in the medium, the limitation of growth is not due to the exhaustion of the supply of either ion by the cells.

Although no net uptake of Na^+ by the cells could be demonstrated by analysis of the supernatant medium, it was still possible that there was Na^+ present in the cells. Even if Na^+ were concentrated to some extent by the cells, the amount of Na^+ removed from the medium relative to the total present would still be so small that the error inherent in the analytical method used would not permit the detection of the uptake if the analysis was applied to the medium. To determine whether or not Na^+ and K^+ were taken up by the cells, and if so what the relative concentrations of the ions in the cells and in the medium might be, the following experiments were performed. Cells were harvested from a medium by centrifuging at $20,000 \times G$ in a Servall angle-head centrifuge. The cell pad obtained was drained, weighed, ashed and analyzed for its content of Na^+ and K^+ . The supernatant medium was diluted appropriately and its Na^+ and K^+ content determined in the flame photometer directly. In order to express the concentrations of both fractions in terms of weight per unit volume, the assumption was made that the specific gravity of the cell pad was one. Since the specific gravity of the cells is slightly greater than one, the concentrations in the cell pads were actually slightly higher than the results obtained would indicate. The ratio of the concentration of each ion in the cell pad to its concentration in the supernatant medium was calculated. From the ratios obtained one can conclude whether the concentration of the ion in the cells is equal to, less than, or greater than that in the medium depending on whether the ratio is equal to, less than, or greater than one (Robertson et al., '55). The ratios obtained when organism B_{16} was grown in the presence of various levels of Na^+ and K^+ are shown in table 5. In each case a crop of cells harvested from 200 ml of medium was analyzed for its Na^+ and K^+ content and each result reported represents the average of five determinations. The organism was grown at three different levels of Na^+ , while K^+ was maintained constant at the level occurring in sea water. It can be seen that when the Na^+ level was 0.64 millimoles/10 ml, which is suboptimal for maximum rate and

extent of growth, the Na⁺ level in the cells was somewhat less than in the medium. When the Na⁺ level reached 1.38 millimoles, which is close to the optimum for growth, the Na⁺ level in the cells and in the medium was approximately the same. At a Na⁺ concentration of 4.19 millimoles per 10 ml which is approximately the level of Na⁺ in sea water, there was less Na⁺ in the cells than in the medium. The corresponding K⁺ ratios show that in all cases K⁺ was at a higher concentration in the cells than in the medium and as the Na⁺ level in the medium increased to the level of Na⁺ in sea water, the inter-

TABLE 5

The effect of medium levels of Na⁺ and K⁺ on the ratio of the concentration of each ion in the cell preparation to its concentration in the growth medium for organism B-16

| MEDIUM LEVELS ¹ | | RATIOS OBTAINED ² | |
|----------------------------|----------------|------------------------------|----------------|
| Na ⁺ | K ⁺ | Na ⁺ | K ⁺ |
| <i>Millimoles/10 ml</i> | | | |
| 0.64 | 0.097 | 0.945 ± 0.015 | 1.333 ± 0.004 |
| 1.38 | 0.104 | 0.978 ± 0.019 | 1.367 ± 0.022 |
| 4.19 | 0.099 | 0.817 ± 0.015 | 1.792 ± 0.088 |
| 1.41 | 2.05 | 0.812 ± 0.005 | 1.031 ± 0.012 |

¹ The medium levels indicated are the starting levels in the uninoculated media.

² The ratios obtained for each ion = concentration of the ion in the cell preparation/concentration of the ion in the medium after growth had ceased.

nal K⁺ concentration increased. When the K⁺ concentration was increased 20-fold to 2.05 millimoles per 10 ml and the Na⁺ level maintained at the optimum level for growth, the K⁺ content of the cells and medium was very nearly the same while the Na⁺ level in the cells dropped from that occurring when the cells grew in a medium containing the same Na⁺ level but a lower concentration of K⁺. The medium having Na⁺ at 4.19 millimoles and K⁺ at 0.1 millimoles per 10 ml contained these ions at approximately the levels at which they occur in sea water. As can be seen, cells from this medium contained less Na⁺ and more K⁺ than the medium.

DISCUSSION

The ratios of the major salts to each other and usually their total concentrations also are strikingly similar in sea water and in the body fluids of animals (Pantin, '31, Dakin, '35). Animal cells contain more K^+ and less Na^+ than is present in the body fluids in which they are bathed. The results reported here indicate that when Na^+ and K^+ are present in the medium at the concentrations occurring in sea water, cells of the marine bacteria studied also contain more K^+ and less Na^+ than is present in the fluid surrounding them. It is thus apparent that mechanisms for the selective elimination of Na^+ and uptake of K^+ occur in marine bacteria as well as in the cells of higher forms of life. Since bacteria are believed to have arisen at an early period in the evolution of life (Oparin and Morgulis, '38), such mechanisms must thus have developed at a very early stage.

Another striking similarity between marine bacterial cells and animal cells is evident when the requirements of both for Na^+ and K^+ are compared. In a study of the salt requirements of two types of mammalian cells for optimum growth in tissue culture, Eagle ('56) has shown the Na^+ requirement to be 110–140 millimoles (when Na^+ supplied by the buffer is included) and the K^+ to be 1–10 millimoles. The corresponding requirements for marine bacteria expressed in the same units are 150–300 millimoles for Na^+ and 1–3 millimoles for K^+ , depending on the organism.

As the need for Na^+ distinguishes marine bacteria from all other bacteria so far investigated with the exception of the halophile *Pseudomonas salinaria* (Brown and Gibbons, '55), the possible relation of marine bacteria to these other organisms is of interest. Halophilic bacteria belong to a rather ill-defined group, some members of which require 15–20 per cent sodium chloride in the medium for growth whereas others, described as moderate halophiles, grow over a much wider range of salt concentration (1–20 per cent) (Baxter and Gibbons, '54). Since the marine bacteria investigated here are

inhibited by the presence of as little as 5 percent NaCl in the medium, it would appear that they can be most readily distinguished from halophiles by their lack of tolerance for high concentrations of salt in the growth medium. Since life is believed to have originated in the sea it is not unlikely that terrestrial bacteria are marine bacteria which have become adapted to growth on land due to the development of an ability to grow without Na⁺ while halophiles are marine bacteria which have developed the capacity to tolerate high concentrations of salt.

The requirement of the organisms for Na⁺ and K⁺ appears to be highly specific. The slight ability of the various alkali-metal ions and sucrose to improve the rate but not the extent of growth of the organisms in the presence of suboptimal concentrations of Na⁺ may be a measure of the degree to which Na⁺ functions to maintain the proper osmotic pressure relations for the cells. The inability of Rb⁺ to substitute for K⁺ in whole or in part for these marine bacteria contrasts with the capacity of this ion to both spare and replace K⁺ in the nutrition and metabolism of various lactic acid bacteria. The antagonism between K⁺ and Cs⁺ demonstrated here, however, has been observed with the latter group of organisms (MacLeod and Snell, '48).

That mechanisms for maintaining K⁺ at a higher concentration in the cells than in the medium are operative in marine bacteria is evident not only from the ratios in table 5 but also from washing experiments. In the latter, Na⁺ but not K⁺ was found to be readily removed from the cells by washing with cold sucrose solutions. Similar findings have been recorded for *E. coli* (Cowie et al., '49). In view of the ability of the cells to concentrate K⁺ it was of interest to find that at levels of K⁺ suboptimal for growth considerable amounts of K⁺ remained in the medium after growth had ceased. To determine how general this phenomenon was, cells of *Lactobacillus arabinosus* were grown in media containing less K⁺ than was required for maximum growth (MacLeod and Snell, '48). Analysis of the supernatant medium after growth had ceased revealed

results essentially the same as those obtained with the marine bacteria.

SUMMARY

For the three marine bacteria investigated, the levels of Na^+ and K^+ in the medium were found to affect both the rate and the extent of growth. Only one one-hundredth as much K^+ as Na^+ was required. Neither Li^+ , Rb^+ nor Cs^+ permitted growth of any of the organisms when added in place of either Na^+ or K^+ . Cs^+ was found to be inhibitory for two of the organisms tested and evidence for antagonism between K^+ and Cs^+ was obtained.

When growth of the organisms was limited by the level of either Na^+ or K^+ in the medium, analysis of the supernatant medium after growth had ceased revealed that considerably more ions remained in the medium than were taken up by the cells.

At suboptimum and optimum concentrations of Na^+ for growth, intracellular and extracellular Na^+ concentrations were nearly the same, while at the level of Na^+ in sea water the extracellular concentration exceeded the intracellular. When K^+ was present in the medium at the level found in sea water the cells contained more K^+ per unit volume than the medium.

Attention has been drawn to the similarity between marine bacterial cells and animal cells as regards both the relationship between intracellular and extracellular Na^+ and K^+ levels and the quantitative requirements of both types of cells for these ions for growth. It has been suggested that both terrestrial bacteria and halophilic bacteria have evolved from marine bacteria, the former by developing an ability to grow without Na^+ and the latter by becoming adapted to growth in the presence of high concentrations of salt.

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THE RELATION OF NATURAL AND IMPOSED ELECTRICAL POTENTIALS AND RESPIRATORY GRADIENTS TO MORPHOGENESIS

REED A. FLICKINGER AND ROBERT W. BLOUNT

*Department of Zoology, University of California
Los Angeles, California*

INTRODUCTION

One of the most puzzling aspects of embryonic development or regeneration is that of morphogenesis or pattern formation: the association of cells to form tissues and organs in the proper topographical relationship to each other in order to constitute the organism. Most of the hypotheses which have been put forth to explain cellular differentiation do not touch upon this problem and usually it is necessary to invoke some type of axial gradient concept (Child, '46; Lund, '28, '47) in an attempt to explain morphogenesis. Although the gradient concept has been much criticized, particularly since it may be too simple to account for the complex aspects of development, it is perhaps better than no hypothesis at all.

Recently Marsh and Beams ('52) and Dimmitt and Marsh ('52) have found it possible to reverse the biological polarity of a regenerating planarian by imposing a potential difference across the worm. In this manner, the head end of a cut planarian, which usually regenerates a head, instead forms a tail, and the tail end of the sectioned worm forms a head. Electrical control of morphogenesis has also been demonstrated for hydroids (Lund, '21, '24, '25, '47; Barth, '34), but not for vertebrate eggs or embryos (Gray, '39; Needham, '31), although Marsh and Beams ('46) have obtained orientation of chick nerve fibers in tissue culture by use of direct electric currents.

Numerous measurements of potential differences have been made upon developing amphibian embryos, but those of Burr and Hovlund ('36), Burr ('41) and Burr and Bullock ('41) are particularly pertinent. These results show that characteristic and reproducible potential differences, in terms of magnitude and polarity, can be determined for various parts of the egg or embryo. The surface of the egg was found to be negative to the ambient water, the animal pole was electro-positive to the vegetal pole and the cephalo-caudal potential increased up through larval development. Lund ('28) has attempted to correlate bioelectric potentials and cellular respiration and invoke these as causal factors in normal morphogenesis. He believes that one group of cells may modify physico-chemical processes in adjoining cells depending upon the strength and direction of a polar electromotive force (E. M. F.) across the tissues. His work shows that regions of higher respiration are electro-positive in relation to areas of lower respiration ('28) and he ascribes the bioelectrical polarity of cells and tissues to differences in the ratio of oxidants to reductants at the poles of the tissue. This potential might be either a summation of the potentials of the many oxidation-reduction couples or the potential of one particular couple in the cell membrane. Most physiologists today ascribe these electrode potentials to diffusion or Donnan equilibrium potentials rather than to oxidation-reduction reactions. Lund ('28) and Marsh ('28) point out that the growing apical end of a hydroid stem (*Obelia*) is electro-positive to the stem and there are other examples of this type of correlation, but Barth ('34) has shown that biological polarity does not always correspond to electrical polarity. In two species of hydroids the end of the stem regenerating the hydranth was electro-positive (*Obelia* and *Eudendrium*), but in two different species (*Pennaria* and *Tubularia*) the end regenerating a hydranth was electro-negative to the stem.

There is no doubt that bioelectric potentials are somehow related to respiration (Lund, '28; Blinks and Darsie, '37), but Blinks and Pickett ('40) found that addition of oxidants

and reductants to sea water did not affect the bioelectric potential of *Nitella* cells. Blinks, Darsie and Skow ('38) believe that respiration may maintain a gradient of ions that accounts for the potential, or respiration may maintain the protoplasmic surface thus accounting for the diffusion potential. Ponder and Macleod ('37) demonstrated that lysins (saponin and bile salts) can abolish the potential difference without reducing oxygen consumption.

The method of measuring these potentials with salt bridges contacting cell surfaces probably means those potentials are associated with cell membranes, whether they are oxidation-reduction or diffusion potentials. Korr ('39) has emphasized that membrane potentials may depend on the relative amounts of oxidized or reduced compounds adsorbed on the membrane. That is, one-half of a redox couple may be adsorbed on the membrane while the other is in the internal cell fluid. The presence of absorbents could thus greatly modify a potential, and also the redox potential of the cell interior may be quite different than that of the membrane.

It has been shown (Korr, '39) that it is not necessary to have noble metal electrodes in order to measure redox potentials. The use of two Ag-AgCl electrodes, with salt bridges leading to points on the organism, gives electrode potentials which can be measured on a galvanometer. It is usually inferred that this implies the movement of ions in the tissue cells towards the two electrodes and electron movement in the external circuit (wire and galvanometer). It is interesting to speculate that the cell membrane might act as the "external circuit" in the organism itself, and perhaps that is not impossible since Geissman ('49) has hypothesized that proteins can transmit electrons through hydrogen bond bridges between polypeptides.

Child ('46) believed that quantitative differences in metabolism along the anterior-posterior, dorsal-ventral, and medio-lateral axes of developing embryos or regenerating organisms could account for the qualitative heterogeneity of organogenesis and morphogenesis. By the use of oxidation-reduction

dyes such as Janus green he has claimed that axial oxidation gradients exist in hydroids and flatworms, in developing salamander embryos, and a number of other organisms. However, Lovtrup ('53) was not able to discern a cephalocaudal gradient of oxygen consumption in planaria when respiration of various regions of the worm were measured using the Cartesian diver technique. By use of quantitative respirometry Barth ('42) has confirmed the existence of respiration gradients in the amphibian egg and embryo.

In the light of these data relating bioelectric potentials and respiration to morphogenesis, it was decided to reinvestigate this problem utilizing frog eggs and embryos and planaria as the experimental organisms.

MATERIALS AND METHODS

The application of polar E. M. F. across eggs and the measurement of bioelectric potentials were carried out utilizing a Rubicon high precision type B potentiometer, a Rubicon spotlight galvanometer, with a sensitivity of $5 \times 10^{-3} \mu\text{a/mm}$ Ag—AgCl electrodes prepared by the method of Burr et al. ('36) and saline-agar bridges (0.9% NaCl—1% Agar). A Burr type electrometer ('36) utilizing Victoreen 5803 tubes was at first used for potential measurements, but later null type measurements with the Rubicon potentiometer were found to be quite adequate. When using early stage frog eggs (*Rana pipiens*), the two salt bridges always made direct contact with the vitelline membrane, while with the later stage frog embryos or with the planaria (*Planaria dorotocephala*) one movable bridge contacted the organism and the other fixed electrode was used as a reference electrode in the solution. When animal-vegetal pole P. D. measurements were made upon frog eggs, or when an E. M. F. was applied across this axis of the frog egg, the egg was allowed to rest upon a fixed saline-agar bridge of a size sufficient to cover a good part of the vegetal hemisphere, while the other bridge was adjusted with a micromanipulator so as to enter the water solution and contact the top half of the egg. For dorso-ventral

measurements, or E. M. F. application, the egg contacted a fixed bridge on one side and a movable "L" shaped bridge on the other side. Jelly was cut from the eggs, but the vitelline membrane was not removed from the early stages. At the gastrula stage this membrane can be removed and measurement revealed similar polar differences of potential for gastrulae with or without membranes, hence the membrane does not appear to mask the true potential at earlier stages.

The E. M. F. measurements upon planaria were made with the worms in distilled water for a brief period. This short exposure to distilled water was not injurious and it served partially to immobilize the worm and reduce shunting of the animal's potential.

Respiration measurements were made utilizing the method of platinum electrode polarography (Davies and Brink, '42). Platinum wire (.020 inch diameter) was fused into glass melting point tubing (.030 inch diameter) so as to allow a short recess (0.5–1.0 mm) at the tip. This electrode was put into a conducting solution (0.6% NaCl) which was part of a circuit consisting of a saline-agar bridge leading to a calomel half cell, a 1.5 volt dry cell, and a Leeds and Northrup type R galvanometer with a sensitivity $4 \times 10^{-4} \mu$ amps/mm. By means of a 500 ohm wire wound potentiometer a voltage of 0.5 volt, when impressed upon the platinum electrode, would reduce the dissolved oxygen at the platinum surface to H_2O_2 and the amount of current measured in microamperes is proportional to the amount of oxygen dissolved in the recess near the platinum. Hence, respiration is inversely proportional to the current readings. Since absolute measurements of oxygen tension were not desired here, the electrode was not calibrated with known amounts of oxygen. However, qualitative tests were made which proved that the electrode was responding to the dissolved oxygen.

In practice the electrode was attached to the low power objective of a dissection microscope and the electrode was placed at the site on the planarian, or egg or embryo, where the respiration measurement was desired by focusing the

microscope. The electrode was usually left on the tissue for a ten-minute period, the voltage was then applied for thirty seconds and the current reading made. The electrode was then removed from the tissue and allowed to refresh itself (oxygen diffusing back into the recess) for a half hour period. No attempt was made to determine the absolute amount of respiration as our interest was only to ascertain the presence of respiratory gradients and their correlation with bioelectric differences. The measurements upon eggs and embryos, and some of the planarian determinations, were conducted with the organisms placed in the bottom of a small paraffin lined petri dish containing 0.6% NaCl. The movement of the planaria necessitated immobilizing them in 1% agar-0.6% NaCl and making the respiratory measurements through holes in the agar at various levels of the worm's body.

The great advantage of this method of respirometry for our purposes was that respiratory rates of small regions of the intact egg, embryo or worm could be measured without cutting the animal into pieces. Child's ('48) work with the redox dyes showed a respiratory gradient in the flatworm and even though the Cartesian diver respirometry of Lovtrup ('53) on parts of the worms apparently disproves this, the latter method is open to several criticisms. One objection is that cutting the worms probably increases respiration at the wounded regions, perhaps thereby obliterating the gradient. A theoretical objection is that the gradient can only exist when the planarian head is present to organize and dominate the metabolism of the active worm and sectioning would destroy the gradient. The use of the platinum electrode polarograph permits the measurement of regional respiration without injuring the intact organism. According to either Child ('46) or Lund ('28) this gradient depends on the organism being intact. Another example where the "whole may be greater than the sum of it's parts" is seen from the work of Grobstein ('52) where a large piece of mouse embryonic shield will differentiate into nervous tissue, but smaller pieces will not.

In some cases the polarograph readings were made while a potential was being applied across a planarian, but this was essentially a combination of the methods outlined above. However, tap water was used in place of 0.6% NaCl in order to avoid the solution shunting current around the animal.

EXPERIMENTAL

The results of the measurements of potential differences in the frog eggs and embryos are summarized in table 1. The potential difference values measured upon frog embryos and planaria varied with the external solution. Potentials were

TABLE 1
Potential difference measurements

| NO. OF MEASUREMENTS | ANIMAL AND DEVELOPMENTAL STAGE | ELECTRODE LOCATIONS | *POTENTIAL DIFFERENCE |
|---------------------|--|---|-----------------------|
| | | | <i>mv</i> |
| 15 | Rana early cleavage | animal-vegetal pole | 2.0-3.0 |
| | | gray crescent-equator | |
| | | opposite | 0.5-1.0 |
| 10 | Rana late blastula (including one with membrane removed) | animal-vegetal pole | 1.0-2.0 |
| | | gray crescent-equator | |
| | | opposite | 0.1-0.5 |
| | | animal pole-gray crescent | 0.0-0.5 |
| 10 | Rana early gastrula | animal-vegetal pole | 0.6-1.0 |
| | | dorsal lip-side opposite | 0.2-0.6 |
| 10 | Rana neurula | neural fold (head region)- neural fold (tail region) | 0.0-0.5 |
| | | neural tube (mid-region)- ventral belly | 0.3-0.5 |
| 5 | Rana tailbud | optic vesicle-neural tube (tail region) | 0.1-0.5 |
| | | neural tube (mid-region)- ventral belly (mid-region) | 0.5-1.0 |
| 2 | Triturus tailbud | optic vesicle-neural tube (tail region) | 2.0-3.0 |
| | | anterior somite-middle somite | 3.0 |
| | | middle somite-posterior somite | 2.0 |
| 20 | Planaria | head-tail | 0.0 |
| | | head-middle region | 2.0-3.0 |

* Values given here refer to the electro-positivity of the first listed electrode site as compared to the second (e.g., animal pole is 2-3 m.v. positive to the vegetal pole.)

higher in distilled water than in tap water and higher in tap water than in saline. This is probably due to increased shunting with solutions of increasing salinity. However in any given solution the variability was probably not greater than 0.1 mv. and since we were interested in comparing regional potential differences and not absolute magnitudes this accuracy was deemed sufficient.

No difficulty with liquid junction potentials was encountered since the electrodes did not penetrate the cells and were in the same solution. Any asymmetry between the two silver-silver chloride half cells was balanced before potential measurements were made.

Like Burr and Bullock ('41) our results show that the external surface of the egg is always negative to the tap water around the eggs and also that the animal pole is electro-positive relative to the vegetal pole. This difference in potential along the future anterior-posterior axis averaged out to three millivolts in the early and middle cleavage stages, but dropped to about 0.6–1.0 m.v. by the gastrula stage. Unlike Burr's ('41) results our determinations showed the region just above the dorsal lip at the early gastrula stage to be electro-positive (0.2–0.6 m.v.) to the corresponding region on the future ventral side. In the neurula the anterior neural plate was 0.1–0.5 m.v. positive to the posterior part of the plate, and the mid-neural plate was 0.3–0.5 m.v. positive to the ventral belly region. By the time the tailbud embryos hatch (stage 18) the head is about 0.5 m.v. positive to the tail, while the dorsal nerve tube is approximately 0.6 m.v. positive to the ventral belly surface. It is interesting that the potentials measured from the ectodermal flank of a hatched larva do not vary significantly when the same area of ectoderm is removed and the reading is made by placing the search electrode directly upon the exposed lateral plate mesoderm. Tailbud salamander embryos (*Triturus torosus* or *Taricha torosa*) show even more striking differences than *Rana pipiens* eggs. The head was 2.5–4.0 m.v. positive to the tail and 7–8 m.v. positive to the ventral gut area. The anterior somites

were 3 m.v. positive to the trunk somites which in turn were 2 m.v. positive to the somites of the tail region.

In an attempt to alter morphogenesis eggs were placed between 0.9% salt bridges in a tap water medium and the normal bioelectric potential differences were reversed. The salt bridges made contact with the animal and vegetal poles, or with the future dorsal and ventral sides of the egg, and the Rubicon potentiometer was used to apply potentials across the egg along these two axes. Usually the eggs were placed between the bridges at the 2-cell stage, or as fertilized, uncleaved eggs, and allowed to develop to the gastrula stage. This means the eggs were subjected to the reversed potential for 24–30 hours. Varying field strengths were applied to the egg, ranging from just reversing the natural potential of the egg to much higher reversed potentials (10, 50, 100, 500, 750, 1,000 and 1,500 m.v.). In all some 59 such experiments were carried out, but the majority of these eggs developed normally and our conclusion is that these applied potentials had no effect. In a few cases abnormal development ensued, but these results could be explained by mechanical injury or salt effects. Although the potential applied to the egg, in a direction opposite to the natural potential, was many times greater than the natural potential, this does not insure that the potential of the egg is reversed. Barth ('34) found that the reversal of the hydroid's natural potential was maintained for some time after the applied potential was discontinued. However, even after 24 hours under the reversed potential, our measurements showed that the eggs possessed polar potentials much like that of control eggs.

At this time it was decided to use another approach, namely, the use of various metabolic poisons, or a salt such as KCl, in the bridges. In this manner it was hoped that alterations in developmental pattern might be obtained by altering reactions which generate bioelectric potentials.

A number of metabolic poisons (NaCN , NaN_3 , urethane, monoiodoacetamide and 2-4 dinitrophenol) were employed in various concentrations in 1% agar bridges, but among

these compounds dinitrophenol proved most satisfactory in giving biological effects. In all, 48 such experiments were made with the metabolic poisons. Cyanide and azide (.01 M) were found to cause marked cellular damage before altering the polar potential difference and it was felt that effects of molecular DNP could be better estimated than those of ionic cyanide or azide. An effect on the potential could then be referred to the metabolic poison, and not to an ionic stimulus. Although .0001 M dinitrophenol is without effect, a .001 M dinitrophenol bridge caused cleavage retardation when applied against the vitelline membrane of a cleavage stage for a 12-hour period. If the egg is then put in tap water, it resumes development, but it is much retarded compared to the controls. A higher concentration of D.N.P. (.03 M), when applied to the animal pole for 4-8 hours caused cleavage retardation but the P.D. remained similar to the controls. However, respiration measurements (platinum electrode polarography) showed equal respiration for both the animal and vegetal poles of the D.N.P. treated egg and evidently the normal respiratory gradient has been obliterated. There is no apparent injury at this time, merely cleavage retardation. This concentration of D.N.P. (.03 M) applied overnight to the animal pole or gray crescent caused cleavage retardation and blockage of development, and these eggs usually do not recover when placed back into tap water. These blocked embryos show drastic inhibition of respiration at the site of D.N.P. contact as revealed by the polarograph. By this time the potential at the treated area is more negative than elsewhere on the egg, but this probably is an injury potential by this time. In no case did D.N.P., or any other metabolic poison, when applied locally to the equator of the egg alter the site of invagination from that dictated by the grey crescent area. Although 2-4 dinitrophenol may first retard cell division and embryonic development, and then affect cellular respiration, the potential is unaffected by these compounds during these same periods. However it is known that bioelectric potentials depend on cellular metabolism and indeed

the potentials measured here fall during later phases of developmental blockage when irreversible injury effects appear.

Since the potential was not closely associated with cleavage and respiration, it was decided to determine the effect of a salt (KCl) upon the potential. The cells of the embryo are probably comparable to adult vertebrate muscle and nerve cells in having a high potassium and low sodium content inside the cells and low potassium and high sodium in the

TABLE 2
Effect of local KCl applications upon bioelectric potential

| DEVELOPMENTAL STAGE | TIME AFTER APPLICATION OF KCL BRIDGE | *POTENTIAL OF ANIMAL TOWARDS DISTILLED WATER |
|------------------------|--|--|
| | <i>min</i> | |
| Rana early cleavage | 0 | 9.0 mv - |
| | 1 | 10.0 |
| | 3 | 11.0 |
| | 5 | 12.0 |
| | 10 | 14.0 |
| Rana early gastrula | 0 | 7.0 mv - |
| | 1 | 10.0 |
| | 2 | 11.0 |
| | 4 | 12.0 |
| | 9 | 15.0 |
| Rana yolk plug | 0 | 4.5 mv - |
| | 3 | 10.0 |
| | 5 | 12.0 |
| | 10 | 13.0 |

* All readings were electro-negative to the distilled water.

intercellular environment. By applying a local source of high potassium concentration at the surface of the embryo by means of a KCl agar bridge, while the embryo is in distilled water to prevent shunting, the potential should decrease if the P.D. is produced by a mechanism similar to that in muscle and nerve cells. When a KCl bridge is applied to the surface of a yolk plug stage frog embryo the potential becomes more negative (table 2) within a few minutes. This depolarization is not an injury potential since the embryos showed no sign of injury from this short salt treatment and their development was completely normal upon being returned to tap water.

This method of affecting the bioelectric potential was used in order to determine if developmental patterns could be altered. Agar bridges with varying concentrations of KCl (0.1–0.5, 1.0, 2.3 M) were placed against the grey crescent of developing eggs. At the higher KCl concentrations local cytolysis occurred at the site of bridge contact, but with the noninjurious concentrations of KCl, there were no biological manifestations and invagination always occurred at the grey crescent site. However, it must be remembered that here there is no assurance that polar bioelectric potentials were actually reversed. Even though short exposures to KCl bridges can markedly alter the potential at that site, longer exposures undoubtedly result in diffusion of KCl and shunting which obviates the effect of the KCl bridge.

The technique of platinum electrode polarography was utilized in order to map respiration rates of various areas of developing frog eggs and embryos. These data were useful in ascertaining the presence of respiratory gradients and also in correlating these gradients with polar bioelectric potentials. The results are summarized in table 3 and they are presented as galvanometer reading in millimeters where the lower readings indicate greater oxygen consumption. These have no absolute value, as our interest was to ascertain the presence of respiratory gradients, not to obtain volumetric determinations of oxygen consumption. Since different recessed electrodes were used in this phase of the work over a year's time, and since no attempt was made to use recessed electrodes with similar volumes, comparison of readings between different experiments is impossible except in terms of gradients and relative trends. However, the readings in any one given set of experiments were made with the same electrode and hence reflect regional rates of oxygen consumption. From the data of table 3, it is clear that the animal hemisphere of the cleaving frog egg has a higher respiration than the equator of the egg which in turn respire more than the vegetal hemisphere. Aside from this animal-vegetal gradient, there is a slight dorsal-ventral gradient with the grey

crescent area (future dorsal side) having a slightly greater oxygen consumption than the corresponding area on the opposite side of the egg (future ventral side). These same respiratory gradients persist up to gastrulation and confirm the work of Barth ('42) who obtained similar respiratory gradients with isolated parts of the embryo using a micro-Winkler technique. At the neurula stage an anterior-posterior gradient of respiration exists along the open neural plate and any region of the neural plate has a higher level of oxygen consumption than the lateral flank or belly region. These

TABLE 3
Platinum electrode polarograph respiration measurements

| NO. OF MEASURE- MENTS | ANIMAL AND DEVELOPMENTAL STAGE | LOCATION ON ANIMAL OF MEASUREMENT | *RELATIVE RESPIRATION |
|-----------------------------|---|--|--------------------------|
| | | | <i>mm</i> |
| 5 | Rana early and middle cleavage stage | Animal pole | 10 |
| | | Vegetal pole | 14 |
| 7 | Rana late blastula and early gastrula | Animal pole | 9 |
| | | Vegetal pole | 13 |
| | | Gray crescent or dorsal lip | 10 |
| | | Equator opposite from dorsal lip side | 11 |
| 2 | Rana gastrula with vitelline membrane removed | Dorsal lip | 10 |
| | | Ventral lip | 13 |
| | | Animal pole | 11.0 |
| 10 | Rana neurula | Head neural plate | 10 |
| | | Mid neural plate | 11 |
| | | Tail neural plate | 12 |
| | | Mid ventral surface | 14 |
| 5 | Rana tailbud | Optic vesicle | 10 |
| | | Pronephric swelling | 10 |
| | | Mid flank | 12 |
| | | Ventral flank | 14 |
| 3 | Triturus tailbud | Optic vesicle | 10 |
| | | Gill swelling | 10 |
| | | Neural tube (tail region) | 12 |
| | | Lateral flank | 13 |
| 20 | Planaria | Head | 10 |
| | | Middle | 10 |
| | | Tail | 10 |

*Galvanometer deflection in millimeters. Values are to be compared only in any given series. Lower values indicate greater respiration.

same anterior-posterior and dorsal-ventral gradients are still present in tailbud embryos of *Triturus* or *Rana* and it was even possible to discern that organ rudiments such as the gill plate respired more than surrounding tissue areas. In comparing the respiration data with the bioelectric results it can be seen that regions of higher respiration are usually electro-positive to areas with less respiration. This is the same correlation that Lund ('28) pointed out, but of course it does not mean that respiration generates these potentials. One typical series of respiration measurements upon planaria is presented in table 3, and it is seen that there is no respiration gradient as claimed by Child, thus confirming the work of Lovtrup ('53) who measured the respiration of isolated pieces of planaria in Cartesian diver respirometers. Apparently then the "whole is *not* greater than the sum of its part," in the sense that a respiratory gradient might exist while the animal is whole, but not when it is sectioned.

A few of some 200 separate potential difference measurements are represented in table 1 showing there is no difference in bioelectric potential between head and tail of starved planaria and also that in many of the flatworms the middle pharyngeal region is somewhat electro-negative to the head and tail. The presence of electro-negativity in the mid-region should mean lower respiration in this area according to Lund ('47) and our failure to observe this difference is apparently an exception to the general correlation of higher respiration with electrical positivity.

As a final check of Lund's theory, it was decided to combine techniques and measure regional respiration while an electrical field was applied along the anterior-posterior axis of the flatworm. The animal was embedded in a small tap water-agar block, and saline-agar bridges were brought down upon the head and tail regions. A voltage was applied from a potentiometer and a constant current was maintained during a given experiment. In a few cases, regional respiration measurements were made with the field polarity in one direction and then, utilizing the same animal, the polarity was

reversed and respiration measurements repeated. The data (table 4) show that in the absence of an externally applied potential there is no significant respiration gradient. The lower voltages (biological range) applied across the long axis of the flatworm did not affect respiration. However,

TABLE 4
Effect of applied currents on regional respiration of Planaria

| EXPT. | POLARITY OF APPLIED CURRENT | REGION | *RESPIRATION <i>mm</i> |
|-------|---|--------|---------------------------|
| 1 | Control (no current) | head | 6.0 |
| | | tail | 6.1 |
| | 50 μ a applied (head end towards the cathode) | head | 4.8 |
| | | tail | 6.3 |
| 2 | Control (no current) | head | 5.3 |
| | | tail | 5.3 |
| | 50 μ a applied (tail toward the cathode) | head | 5.5 |
| | | tail | 4.2 |
| 3 | Control (no current) | head | 6.2 |
| | | tail | 6.1 |
| | 50 μ a applied (tail toward cathode) | head | 7.9 |
| | | tail | 6.1 |
| | Reversed polarity of applied current (head toward the cathode) 50 μ a applied | head | 7.1 |
| | | tail | 9.1 |
| | Same case as above 12 hours after cessation of current application | head | 9.3 |
| | | tail | 9.2 |

* Galvanometer deflection in millimeters. Readings in a given expt. were taken at 40-minute intervals unless stated otherwise.

when the animal is subjected to higher applied potentials and current (only the current was measured in these experiments), the end of the animal facing the cathode apparently respire more rapidly than the end facing the anode. This induced respiratory gradient was then reversed by reversing the polarity of the applied current (potential is also reversed). In order to eliminate the possibility that an applied current

was interacting with the respirometer, a control experiment was performed. The planarian was replaced by a rectangle of saline-agar, while all other conditions were maintained as above, and readings were taken with the recessed electrode placed at either end of the agar block in order to simulate experimental conditions. No variations in polarograph readings occurred as a function of position of the recessed electrode, the polarity of the applied current, or the presence or absence of the applied current. The magnitude of the applied potential, which was capable of producing a respiration gradient, was of approximately the same order as that used by Marsh in directing axial regeneration in cut planaria, and hence the mechanism of the latter transformation may possibly be due to the production of a respiratory gradient.

DISCUSSION

Although there is a correlation between higher respiration and more positive bioelectric potential in the amphibian egg and embryo, this does not hold for the mid-region of most of the planaria. The use of metabolic poisons also provides a means of dissociating respiration and potentials. In these experiments short exposure to localized application sites of 2-4 dinitrophenol were found to cause cleavage retardation, without affecting respiration and potential, longer exposure resulting in reversible biological blockage caused inhibition of respiration with no change in potential, and finally application of the metabolic poison for periods which resulted in irreversible developmental blockage and injury gave marked inhibition in oxygen consumption and more negative potential measurements. The striking depolarization caused by short periods of application of KCl bridges (table 2) to frog eggs is perhaps the best evidence that bioelectric potentials have their primary origin apart from oxidation-reduction processes. The appearance of more negative potentials at the site of KCl contact occurs within a few minutes after contact; there is no obvious injury and development proceeds normally

when the eggs are returned to tap water, thus this is apparently not an injury potential.

The fact that applied potential and current within the biological range did not affect respiration also argues for the fact that bioelectric potentials are not directly associated with respiration, although obviously the two are related in some ways. The apparent stimulation of respiration by higher reversed potential and current is difficult to explain.

One of the possible reasons that applied potentials did not interfere with the development of the frog egg is that there already exists within the egg a structural heterogeneity, e.g., yolk gradient, that prevails over the applied E.M.F. In the case of hydroids (Lund, '21, '23a; Barth, '34) and planaria (Marsh and Beams, '52), where the axial polarity of regeneration can be electrically controlled, selective inhibition may be operating upon an essentially bi-potential system since it is known that a head or hydranth can form at any level of a cut planarian or hydroid.

Another mechanism which undoubtedly, at least partially, accounts for the proper positioning of cells, tissues and organs in morphogenesis is the selective affinities existing between cell surfaces. This is demonstrated most strikingly by the reunion of dissociated amphibian embryo cells (Townes and Holtfreter, '55). Spiegel ('54) has inhibited this reunion by antiserum treatment and thus emphasized the role that proteins of the cell surface may play in this process. Although selective cell and tissue affinities may possibly explain tissue and organ formation, it is more difficult to discern how these affinities might account for the regular placement of the various organs so as to provide the normal body plan, rather than a chaotic association of tissues and organs.

SUMMARY

1. Bioelectric potentials were recorded from the head, mid- and tail regions of planaria and from various regions of developing frog eggs and embryos. A number of charac-

teristic and reproducible polarities were found for the amphibian egg and embryo, but no electrical polarity was detected in planaria.

2. Polarographic respiration measurements were made with recessed platinum electrodes at the same regions of intact planaria and frog eggs and embryos at which the E.M.F. measurements were obtained. Respiration gradients could not be detected in the flatworms but a number of gradients were mapped in the frog egg and embryo.

3. By means of dissolving various concentrations of the metabolic poison 2, 4-dinitrophenol, or a salt (KCl), in the agar of saline-agar bridges, local applications of these compounds to various regions of developing amphibian eggs was possible. Dinitrophenol was found first to affect development by causing cleavage retardation while the respiration and potential were unaffected. Longer application of dinitrophenol caused some inhibition of respiration, although the potential remained normal. Marked inhibition of respiration and potential were seen in cases of irreversible developmental blockage and this more negative potential may be due to an injury potential. The immediate depolarization (i.e., potential becoming more negative) caused by local application of KCl suggests that the bioelectric potential may be primarily controlled by distribution of ions across membranes.

4. Although regions of greater respiration were usually electro-positive to sites of lower respiration, this correlation did not exist in the planaria, where the mid-region was usually electro-negative to the head and tail, although these three regions have equal rates of respiration. The dissociation of respiration and potential by means of 2-4 dinitrophenol and the marked lowering of potential by KCl argues for only a distant correlation between oxidation-reduction processes and bioelectric potential.

5. When an E.M.F. is applied along the long axis of a planarian and respiration determinations carried out simultaneously, it was discovered that at the higher imposed potentials and currents respiration was greater at the cath-

odal pole than at the anodal pole, thus a respiration gradient was created in the flatworm. Although the evidence cited in 4 above argues that potentials are not directly generated by oxidation-reduction reactions, the respiration data from planaria under an applied current and potential imply that bioelectric potentials may possibly affect respiration.

6. When frog eggs were allowed to develop in a potential field which was opposite to their normal bioelectric polarity, there was no alteration in the normal developmental pattern.

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DDT AND THE HEMOCYTE PICTURE OF THE MEALWORM, *TENEBRIO MOLITOR* L

JACK COLVARD JONES ^{1,2}

*U. S. Department of Health, Education, and Welfare, Public Health Service,
National Institutes of Health, National Institute of Allergy and Infectious
Diseases, Laboratory of Tropical Diseases, Bethesda 14, Maryland*

In a previous study it was shown that DDT did not lyse hemocytes (or blood cells) of the mealworm *in vitro* (Jones and Tauber, '54), although Toumanoff and Lapied ('50) reported this to be the case in DDT-treated *Galleria*. In addition, it was shown that DDT did not interfere with *in vitro* hemolymph coagulation in the mealworm (Jones and Tauber, '54), although Grégoire ('53) found inhibition of coagulation in hemolymph from some insects after adding 10% DDT suspensions to it. Finally, it was concluded that abnormal hemocytes in DDT-poisoned mealworms did not result from a direct toxic action of the poisons on these cells but were a wholly secondary phenomenon.

Toumanoff and Lapied ('50) reported that marked changes occurred in differential hemocyte counts of DDT-poisoned *Galleria*. Specifically, they stated that contact with, or injection of, DDT brought about a "destruction of true leucocytes or micronucleocytes" and that there was an increase in "leucocytes with azurophile granules" (= plasmatocytes in present study). They interpreted this increase as a defense reaction.

The present study was designed to see if a comparable phenomenon would take place in the hemocyte picture of mealworms poisoned with DDT.

¹ Present address: Department of Entomology, University of Maryland, College Park, Md.

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METHODS

Large larvae (25 to 30 mm long) of *Tenebrio molitor* L. (Coleoptera) were used for all studies. The experiments were conducted in a room held between 25 and 28°C with a relative humidity varying between 50 and 70%. Larvae were exposed to pure, finely powdered p,p' DDT in glass beakers. Hemocytes were examined in unfixed hemolymph (Jones, '54) and following heat fixation of the whole insect for two minutes in a water bath held at 55°C. Cells in Giemsa stained films from heat-fixed specimens were identified by a classification given by Jones ('50). Cells in wet spread coverslip preparations from unfixed and heat-fixed material were classified under the phase microscope as previously described (Jones, '54). Total hemocyte counts were made according to the method of Jones and Tauber ('51). Blood volumes were determined by a slight modification of the amaranth red dye technique given by Yeager and Munson ('50).

RESULTS

Differential hemocyte counts made on Giemsa stained blood films from heat-fixed DDT-treated and control larvae showed that throughout the 7-day sampling period the percentages of cells identified as plasmatocytes³ were consistently and significantly higher in larvae exposed to DDT (table 1). Significantly greater variation was found in hemocyte pictures of heat-fixed DDT-treated larvae than in those of heat-fixed controls.

Percentages of plasmatocytes were higher in convulsing (52%) than in non-convulsing (44.7%) heat-fixed DDT-treated larvae. Plasmatocytes accounted for about 72% of the hemocytes in heat-fixed shrunk and dehydrated (moribund) larvae. No significant differences were found between hemocyte

³ Plasmatocytes are phagocytic hemocytes in the mealworm. In heat-fixed blood films stained with Giemsa, they are ovoid or fusiform cells with a blue cytoplasm and purple nucleus. Their cytoplasm generally contains fine red granules. In unfixed and unstained wet blood drops and films the plasmatocytes tend to develop pseudopodial extensions.

pictures of pre-molt and post-molt heat-fixed DDT-treated larvae.

Among 40 mealworm larvae exposed throughout a week to DDT, the total number of hemocytes (cells/mm³) remained within normal limits; that is, the number varied from 41,000 to 69,000 (mean of 50,400) cells/mm³. Total counts could not be made from moribund larvae due to scarcity of hemolymph. Prior to this stage there was no marked change in blood volume (blood volume of DDT-treated larvae = 9.6% body

TABLE 1
Changes in plasmatocyte percentages of DDT-treated and control mealworm larvae

| TREATMENT | NO. LARVAE USED | TYPE PREPARATION | % CELLS IDENTIFIED AS PLASMATOCYTES |
|---------------------|-----------------|---|-------------------------------------|
| DDT, 24 - 168 hours | 200 | { Larvae heat-fixed 2 minutes at 55°C; Giemsa stained films | 48.2 ± 1.1 |
| Controls | 175 | | 42.2 ± 1.0 |
| DDT, 24 - 192 hours | 60 | { Unfixed drops and films examined with phase microscope | 49.9 ± 0.6 |
| Controls | 60 | | 54.8 ± 0.6 |
| DDT, 24 hours | 10 | { Unfixed; phase Heat-fixed; phase | 44.2 59.5 |
| Controls | 10 | { Unfixed; phase Heat-fixed; phase | 51.9 50.3 |

weight; controls = 10%), even though DDT-treated larvae lost two to four times more weight and lost it faster than starving controls.

Phase contrast differential counts made on *unfixed* hemolymph taken from living DDT-treated and control larvae differed strikingly from the preceding data (table 1), for the plasmatocytes, instead of being higher, were now significantly lower in the DDT-treated than in the control group.

Since plasmatocytes appeared noticeably higher in unfixed controls examined with phase microscopy than in stained blood films from heat-fixed controls examined with ordinary light, it

was necessary to make differential counts from both unfixed and heat-fixed specimens with the phase microscope. Considerable difficulties were sometimes encountered in distinguishing plasmatocytes from cystocytes⁴ in heat-fixed wet preparations under phase, but no such difficulties occurred with unfixed preparations.

While no differences were found between phase differentials of unfixed and heat-fixed controls, cells identified as plasmatocytes in DDT-treated larvae were still low in unfixed and high in heat-fixed specimens. This difference was equally

TABLE 2

Total hemocyte counts in DDT-treated and control mealworm larvae

| TREATMENT | TYPE PREPARATION | NUMBER CELLS/MM ³ | |
|---------------|------------------|------------------------------|---------|
| | | Range | Average |
| DDT, 24 hours | Unfixed | 32,100-37,700 | 34,900 |
| | Heat-fixed | 42,600-49,000 | 45,800 |
| Controls | Unfixed | 20,000-36,000 | 28,000 |
| | Heat-fixed | 42,000-47,000 | 44,500 |

apparent when the same larvae were used for both unfixed and heat-fixed preparations (table 1).

To obtain more information about this phenomenon, total cell counts were made from unfixed and then heat-fixed mealworms. As indicated by the data in table 2, the total count in DDT-treated mealworms increased by about 11,000 cells/mm³ following heat-fixation. By calculation the number of their plasmatocytes increased by 12,000/mm³. Thus, the increase in plasmatocytes more than accounts for the increased total count. On the other hand, total counts in controls, though

⁴Cystocytes are hemocytes which initiate coagulation of the blood in the mealworm. In heat-fixed blood films stained with Giemsa they are round or ovoid cells with a pale blue or grey cytoplasm and a small compact round nucleus. The cytoplasm is filled usually with numerous red granules which are typically larger than those in plasmatocytes. In unfixed and unstained blood drops and films the cystocytes are small round unstable cells with brilliant hyaline cytoplasm and well defined cartwheel-like nucleus. These cells tend to form cytoplasmic blisters in unfixed drops of blood.

more variable, increased by about 17,000 cells/mm³ after heat fixation (table 2); yet the increase in plasmatocytes accounted for less than half (7,500/mm³) of the total number of hemocytes. In other words, while total cell counts for heat-fixed DDT-treated and control larvae were approximately the same, cells identified as plasmatocytes increased *only* in the DDT-treated group.

The preceding data strongly suggested that heat fixation altered some of the cystocytes in such a way that they would be systematically identified as plasmatocytes in differential counts, and that more cystocytes would be thus altered in the DDT-treated larvae than controls. To test this normal mealworms were fixed 2 minutes in water held at 40, 45, 50, 55, and 60°C and blood drops examined under the phase microscope. Plasmatocytes showed only a small increase (1 to 5%) between 40 and 55°C, but showed a very marked increase (25%) at 60°C. DDT-treated larvae showed a marked increase in plasmatocytes at 55°C. It is, therefore, concluded that heat-fixation is capable of altering the hemocyte picture of the mealworm by obscuring the distinguishing differences between the two major kinds of cells so that systematic errors occur and that this effect is most pronounced in those treated with DDT.

The only well known types of defensive reactions of insect hemocytes are (1) increases in phagocytic cells during phagocytosis of foreign bodies with subsequent encapsulation of these, and (2) rapid initiation of hemolymph coagulation after wounding. In the mealworm, plasmatocytes are the primary phagocytic cells (Jones and Tauber, '54), and cystocytes initiate coagulation in the blood (Grégoire, '51).

Normal mealworms injected with ammonia carmine were found to have more plasmatocytes (78.5%) in unfixed blood drops within 24 hours than either saline injected (57.6%) or uninjected larvae (53.7%). About 33% of their blood cells contained carmine granules. This blood picture is obviously very different from that found in DDT-treated mealworms. It has already been shown that hemolymph coagulation in DDT-

treated larvae is the same as that in controls (Jones and Tauber, '54).

The changes in the blood picture during DDT poisoning are so slight prior to the moribund stage as to indicate that hemocytes must play a very insignificant role, if any, in the effective defense of the mealworm.

SUMMARY

The phagocytic hemocytes (plasmatocytes) slightly but significantly decrease during DDT poisoning in unfixed, DDT-treated mealworm larvae and remain so prior to the moribund stage. Heat-fixation of these larvae significantly alters this picture by obscuring the distinguishing features between the two main kinds of cells. Total hemocyte counts (cells/mm³) in unfixed and heat-fixed DDT-treated larvae are within the normal range prior to the moribund stage. On the basis of these findings, it is concluded that hemocytes play no role in the natural defenses of the mealworm against DDT.

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AN ANALYSIS OF GROWTH IN NUCLEAR POPULATION DURING WALLERIAN DEGENERATION

M. ABERCROMBIE AND JOYCE E. SANTLER

Department of Anatomy and Embryology, University College London

INTRODUCTION

During the early stages of Wallerian degeneration in a myelinated peripheral nerve there is an outburst of mitosis. A considerable cell population is built up within a few weeks, probably as a result of the mitotic activity, though some contribution from the blood of mononuclear cells is not yet entirely excluded. There seems to be no reasonable alternative to the view that the stimulant to cell multiplication is provided by the break-up of the nerve fibres, though exactly what the stimulant may be is still a matter for speculation. Abercrombie and Johnson ('46) suggested that the degeneration of the fibres released some diffusible mitotic stimulant. Joseph ('50) on the other hand suggested that, as the nerve fibres disappeared, the vacant space so created stimulated mitosis.

The present paper is concerned, not with the nature of the stimulant, but with the nature of the reaction to it: it is an attempt to analyse what it is that controls the amount of cell population growth in degenerating nerves. It is a plausible assumption that, whatever its nature, quantity of stimulant can be approximately represented by the quantity of its source, the nerve fibres. The relation between cell population growth and amount of stimulant can therefore be investigated. It would be over-ambitious to attempt to construct a dosage-response curve, but at least the question whether or not there is *any* positive correlation between dosage and response can

be answered. This makes it possible to examine the hypothesis that the stimulant acts merely as a trigger, the total quantity of population growth being limited by something else, most obviously by the increased density of cell population it produces (Joseph, '50). Even if there is no "ceiling" to growth imposed by density, it is still important to investigate the relation between the density of a population exposed to a certain degree of stimulation and the amount of growth that population undergoes. The existence of a negative correlation would raise the possibility that what ecologists call "density-dependent factors" influence population growth.

Peripheral nerve has the great advantage for such an investigation that it can be obtained with a wide range of different nerve fibre contents and of different cell densities. These variations can be found in anatomically different nerves of different fibre spectrum, and this variation has already been exploited for the analysis of cell population growth by Joseph ('48, '50) and Thomas ('48). Such variations can also be artificially prepared within a single kind of nerve in the following way. When a nerve is severed the cell population rises and then falls again (Abercrombie and Johnson, '46; Thomas, '48) but it does not fall back to its normal level even when reinnervation is allowed to occur (Abercrombie, Johnson and Thomas, '49; Logan, Rossiter and Barr, '53). Reinnervated nerves therefore have a higher cell population than normal nerves and a content of nerve fibres which depends on the time elapsed since reinnervation started. Severed a second time, they undergo cell multiplication starting from a range of initial states which can be very different from that found in normal nerves.

The purpose of the present paper is to exploit this method of varying by predegeneration the composition of the nerve in which Wallerian degeneration is to be induced. We are unable by this means to vary the initial density of population and the quantity of nerve fibre independently, and the analysis therefore has to be made by multiple regression. The method has a further complication in that an extra variable is in-

troduced by the different periods of preparatory reinnervation which give the nerves their different compositions. This extra variable, representing the previous history of the cell population, has to be allowed for, but itself repays analysis.

METHOD

The experimental animals were rabbits. Operations were performed under Nembutal and ether anaesthesia. In all animals the sciatic nerve, usually of one side but sometimes of both sides, was prepared as a reinnervated nerve by a first operation at which the nerve was crushed (three or 4 times in quick succession) with smooth-tipped forceps high in the thigh, at approximately the level of the third trochanter. In a nerve so treated reinnervation of the distal stump begins after an interval which averages about 5 days (Gutmann, Guttmann, Medawar and Young, '42). It was allowed to proceed for a variable period. A second operation was performed on all rabbits 25 days before autopsy. In this pre-autopsy operation Wallerian degeneration of both sciatic nerves was produced by cutting them with scissors about $\frac{1}{2}$ cm below the level of the third trochanter, and a piece of the distal stump about 1 cm long was removed for fixation. At autopsy, 25 days later, the proximal $\frac{1}{2}$ cm of each distal stump was discarded because it might have been affected by the trauma of operation, and the next most distal centimetre was fixed for histological study. From each nerve we therefore obtained two pieces for histological comparison, taken before and after the final period of degeneration.

Three different schemes of preparatory operation were used to obtain different periods of reinnervation. Each scheme was applied to a set of 10 or 11 rabbits.

1. In the first set the nerve of only one side was crushed, and it was left for 100 days before the pre-autopsy operation. This group thus provided a group of normal nerves and a group of nerves reinnervated for 100 days (or as near as makes no matter) before degeneration by the pre-autopsy operation.

2. In a second set of animals the same procedure was followed except that 300 days were allowed to elapse between the preparatory crushing and the pre-autopsy operation, thus providing normal and 300-day reinnervated nerves. The latter, as will be shown later, have a considerably greater volume of nerve fibre than have the 100 day reinnervated nerves, but their fibre content is still not quite back to normal.

3. In a third set of animals the nerve of one side was crushed and left for 100 days; then at a second preparatory operation the nerve of that side was crushed for a second time at the same level, and the nerve of the other side was crushed for the first time. At the pre-autopsy operation this group thus provided both 100 day reinnervated nerves and nerves which had been reinnervated twice for 100 days each time.

The group of normal nerves provided by operation schemes (1) and (2) will be referred to as the *0 day group*; the nerves reinnervated for 100 days [operation schemes (1) and (3)] will make the *100 day group*; the nerves reinnervated twice for 100 days [operation scheme (3)] will make the *100 + 100 day group*; and, the nerves reinnervated for 300 days [operation scheme (2)] will make the *300 day group*.

The piece of nerve removed at the pre-autopsy operation was divided into two. One part was fixed in Flemming and the other part, the more distal, in Susa. Sections were cut at $6\ \mu$; transversely and longitudinally in the case of the Susa-fixed material, transversely only in the case of the Flemming-fixed. The piece removed at autopsy was fixed in Susa. The Susa-fixed sections were stained with haematoxylin and eosin for counting the number of nuclei. Those fixed in Flemming were stained by the Weigert method for estimation of the quantity of nerve-fibre material.

Counts of all nuclei in one transverse section were made of three branches of the sciatic, the nervus gastrocnemii medialis (henceforward called the *n.g.m.*), the peroneal branch and the sural branch. Such a count was made in each of the two pieces (pre-autopsy and autopsy) fixed from each nerve.

The crude count of the number of nuclei in each transverse section was then corrected for nuclear size (Abercrombie, '46), the mean nuclear length being obtained for this purpose in each piece of nerve fixed by measuring about 100 nuclei in longitudinal section. The area in transverse section of each nerve was obtained by projection and measurement with a planimeter. The proportion of the area of each transverse section occupied by nerve fibre was estimated on the Fleming-fixed pieces removed at the preautopsy operation by the method of Chalkley ('42). All material within the outer limit of the myelin of each fibre was counted as nerve fibre material.

The relation of three independent variables to three dependent variables expressing growth was determined by multiple regression analysis. The three independent variables were the time between the first, preparatory, operation and the pre-autopsy operation ("*preparatory period*"); the proportion of the nerve occupied by myelinated nerve fibre at the pre-autopsy operation just before the final degeneration ("*fibre proportion*"); and the density of nuclear population at the pre-autopsy operation ("*initial density*"). The three dependent variables were density of nuclear population at autopsy, after the final degeneration ("*final density*"); \log_e of the population at autopsy minus \log_e of that at the pre-autopsy operation ("*specific growth rate*"); and the net number of new nuclei added during the period between the pre-autopsy operation and autopsy, expressed per unit of initial volume ("*increment per unit volume*").

CRITIQUE OF THE VARIABLES

It is important to make clear the main assumptions which have entered into the estimation and interpretation of the variables, so that the validity of the use we put them to can be assessed.

Fibre proportion. Our estimate of the amount of nerve fibre material per unit volume of nerve includes both axon and myelin sheath. The histological procedure is generally

reckoned to keep distortion low (see Sanders, '48; Evans and Vizoso, '51). We lack, however, any data which would enable us to judge our implicit assumption that the distribution of shrinkage between nerve fibres and the rest of the nerve is similar at different times of reinnervation. The histological treatment has a major drawback in that it entails complete neglect of unmyelinated fibres. Since we wish to represent the total mass of nerve fibre material by fibre proportion, we are certainly on this account suffering from an error which will be different in the different classes of nerve. In particular the sural branch has a large content of unmyelinated fibres. But the error is unlikely to be of any importance in artificially creating the observed correlations within any one branch of the sciatic for the following reasons: unmyelinated fibres are not likely to be an important source of stimulus judging by the fact that nuclear population growth is absent (Joseph, '47) or small (Abercrombie, Evans and Murray, unpublished) in unmyelinated nerves; and even if they are a source of stimulus, they are more likely to have obscured than produced the positive relation we found between fibre proportion and growth, because the reinnervated nerves with the smallest fibre proportion would presumably be those with myelination least advanced and hence with the most unmyelinated fibres.

Initial density. Determination of this independent variable requires two basic estimates, nuclear population and cross-sectional area. The nuclear population is an enumeration of all the nuclei within the nerve and therefore disregards any differences in the proportion of different kinds of cells. We know, however, that such differences are likely to occur (Abercrombie and Johnson, '46; Thomas, '48). The result of degenerating a nerve is to increase differentially its content of tubal, probably Schwann, cells. But since the tubal cells undergo the most multiplication during degeneration, the effect of standardising for this difference in population make-up would if anything be to strengthen the correlations observed, by diminishing still further the growth found in the

regenerated classes of nerves. The measurement of area again involves possible error from differential shrinkage.

Growth measurements. We have used three ways of expressing growth, suggested by the need to test certain hypotheses about its control. Two of the growth measurements, specific growth rate and increment per unit volume, involved a comparison within one nerve made on two pieces taken respectively at the time of pre-autopsy operation and 25 days later. This means that different levels of a nerve were compared with each other. Different levels in other nerves are known to have similar nuclear populations (Joseph, '48; Logan, Rossiter and Barr, '53); and all our experience indicates that if there are any differences in the rabbit sciatic

TABLE 1

Mean swelling (final area/initial area) during 25 days degeneration. Numbers of specimens in the groups as in table 2. The nerves are grouped according to branch and period of preparatory reinnervation

| NERVE BRANCH | TIME OF REINNervation, DAYS | | | |
|--------------|-----------------------------|----------------|----------------|----------------|
| | 0 | 100 | 100 + 100 | 300 |
| N.g.m. | 1.09 \pm .06 | 1.00 \pm .06 | 0.99 \pm .09 | 0.96 \pm .04 |
| Peroneal | 1.10 \pm .04 | 1.03 \pm .03 | 1.04 \pm .05 | 1.17 \pm .06 |
| Sural | 1.39 \pm .11 | 1.43 \pm .11 | 1.15 \pm .11 | 1.13 \pm .13 |

they are small ones. Since the level of operation was standardised, any differences of population will be standardised too as between the different reinnervation time groups.

During the first few weeks after it has been cut, a peripheral nerve swells (Abercrombie and Johnson, '46). Such swelling must affect final density of population, and would affect the concentration of any growth promoting substance that might be postulated. It is necessary to know about its occurrence during the degeneration of our reinnervated nerves, and particularly about the existence of any correlations between the amount of swelling on the one hand and fibre proportion and initial density on the other hand. The mean swelling (cross-sectional area of nerve at pre-autopsy operation divided by cross-sectional area at autopsy 25 days later) for the different nerves (table 1) is obviously slight except in

the sural. A multiple regression analysis was applied to these data, taking fibre proportion and initial density as independent variables, and swelling as dependent variable. Treating each branch of nerve separately but pooling the different reinnervation groups, no regression coefficient gave any hint of significance, nor did any of the three multiple correlation coefficients. Combining all the data, but within branches, the partial regression coefficient ($\times 10^3$) for fibre proportion was 5.1 ± 3.2 , which is not significantly different from zero ($t = 1.6$, d.f. 113, $P = 0.1$); and the partial coefficient ($\times 10^3$) for initial density was 1.59 ± 1.56 , obviously non-significant ($t = 1.1$, d.f. 113, $P = 0.3$); The multiple correlation coefficient was only 0.159. The conclusion is that no relation can be detected between amount of swelling during degeneration and the initial make-up of the nerve as we have measured it in our own material.

An important point is that 25 days after pre-autopsy operation is assumed to be a comparable time in the course of the nuclear population changes in the different classes of nerve. This is known not to be so in severed normal nerves of the different sciatic branches. In these, apparently, the time at which the maximum population is reached varies with the mean fibre size (Thomas, '48; Joseph, '50). It is not known whether this relation holds within reinnervated nerves, but it may do so. Our measurements of growth are intended to represent the whole growth attained up to the population maximum. How will they be affected by variation in time of the maximum population? The population at 25 days is probably not far from its maximum in the 0 day groups of n.g.m. and peroneal; it will be beyond its maximum in the sural branch, though judging by its time-course (Thomas, '48) the effect of this will not be serious; and it will also be beyond its maximum in most of the reinnervated nerves insofar as they have the same relation of peak population with mean fibre size. This would mean that the maximum population reached in reinnervated nerves after degeneration will have been underestimated in relation to that of the normal nerves,

and this may have contributed something to the correlations actually found.

Finally we make the assumption that the increase of population is due to a multiplication of indigenous cells. There is at present no good evidence that immigration plays any significant part, though equally it has not been ruled out. Polymorphs are never found in degenerating nerve (excluding the traumatised region) and the passage of lymphocytes or monocytes through vessel walls has not, so far as we are aware, been observed.

The list of possible defects in our data could be extended. It will be shown, however, that by means of the independent variables we have used we can account for 90% of the variance of specific growth rate and 75% of that of increment per unit volume in n.g.m. and peroneal. It seems, therefore, highly likely that no uncorrelated independent variable of importance has been missed in the analysis at least of these two branches. The relative inadequacy of our analysis of the sural branch, and of final density in all three branches, will be discussed later. It remains possible however that we have neglected some important variable strongly correlated with those we have used: in other words that our chosen independent variables represent something other than we suppose. Apart from possibilities discussed already, one such neglected variable which might be important is vascularity. We do not even know with which, if any, of the independent variables we have used it is likely to be correlated.

RESULTS

Results of the counts and measurements are set out in tables 2 and 3 in the form of means of the various groups of nerves. The data for each branch of the sciatic are set out in four groups according to the reinnervation the nerves had undergone before their final degeneration. In table 2 the mean nuclear population (corrected for nuclear length) of one complete transverse section, and the mean area of such a transverse section is given at the pre-autopsy operation

TABLE 2

Means with standard errors of the counts and measurements used for the derivation of population densities and growth rates of table 3. The nerves are grouped according to branch and period of preparatory reinnervation ("Time of Reinnervation"). N = number of nerves

| NERVE BRANCH | TIME OF REINNervation | N | NUCLEAR POPULATION PER SECTION | | CROSS-SECTIONAL AREA, $\text{MM}^2 \times 10^{-2}$ | |
|-----------------|--------------------------|----|--------------------------------|----------------|--|----------------|
| | | | Initial | Final | Initial | Final |
| N.g.m. | 0 | 8 | 34 \pm 2.8 | 513 \pm 29 | 6.6 \pm 0.3 | 7.1 \pm 0.3 |
| | 100 | 11 | 218 \pm 18 | 403 \pm 28 | 8.3 \pm 0.5 | 7.9 \pm 0.5 |
| | 100 + 100 | 6 | 284 \pm 25 | 380 \pm 46 | 8.1 \pm 0.7 | 8.0 \pm 1.0 |
| | 300 | 4 | 174 \pm 14 | 292 \pm 6 | 7.5 \pm 0.7 | 7.2 \pm 0.9 |
| Peroneal | 0 | 17 | 544 \pm 20 | 3035 \pm 161 | 41.7 \pm 1.4 | 46.7 \pm 1.6 |
| | 100 | 16 | 1686 \pm 104 | 2628 \pm 144 | 43.5 \pm 1.5 | 45.0 \pm 7.0 |
| | 100 + 100 | 9 | 2209 \pm 194 | 2716 \pm 312 | 47.0 \pm 3.2 | 50.0 \pm 5.9 |
| | 300 | 9 | 1512 \pm 81 | 2250 \pm 113 | 40.8 \pm 1.3 | 47.4 \pm 2.2 |
| Sural | 0 | 14 | 265 \pm 10 | 911 \pm 69 | 9.7 \pm 0.3 | 13.3 \pm 1.0 |
| | 100 | 15 | 407 \pm 16 | 1055 \pm 56 | 10.5 \pm 1.1 | 15.0 \pm 4.0 |
| | 100 + 100 | 5 | 749 \pm 87 | 964 \pm 119 | 11.1 \pm 0.8 | 13.4 \pm 1.6 |
| | 300 | 5 | 544 \pm 66 | 799 \pm 61 | 11.7 \pm 0.7 | 13.2 \pm 1.8 |

TABLE 3

Means with standard errors of two independent variables (fibre proportion and initial density) and (next three columns) the three dependent variables used. The nerves are grouped as in table 2. N = number of nerves

| NERVE BRANCH | TIME OF REINNervation | N | NERVE FIBRE CONTENT, % | DENSITY OF NUCLEAR POPULATION | | SPECIFIC GROWTH RATE | NUCLEI ADDED PER UNIT INITIAL VOL. |
|-----------------|--------------------------|----|---------------------------|-------------------------------|--------------|----------------------------|--|
| | | | | Initial | Final | | |
| N.g.m. | 0 | 8 | 82.0 \pm 2.4 | 29.4 \pm 2.0 | 421 \pm 26 | 2.73 \pm .10 | 428 \pm 33 |
| | 100 | 11 | 51.0 \pm 2.4 | 152 \pm 10 | 313 \pm 37 | 0.63 \pm .11 | 139 \pm 28 |
| | 100 + 100 | 6 | 50.4 \pm 2.4 | 208 \pm 21 | 280 \pm 24 | 0.27 \pm .05 | 68 \pm 16 |
| | 300 | 4 | 70.4 \pm 2.2 | 135 \pm 27 | 243 \pm 24 | 0.53 \pm .08 | 112 \pm 20 |
| Peroneal | 0 | 17 | 81.3 \pm 0.7 | 74.5 \pm 2.2 | 387 \pm 23 | 1.69 \pm .06 | 335 \pm 20 |
| | 100 | 16 | 52.7 \pm 1.2 | 222 \pm 12 | 334 \pm 13 | 0.44 \pm .05 | 132 \pm 13 |
| | 100 + 100 | 9 | 45.2 \pm 2.7 | 267 \pm 14 | 311 \pm 14 | 0.18 \pm .04 | 55 \pm 13 |
| | 300 | 9 | 67.6 \pm 1.5 | 212 \pm 12 | 273 \pm 15 | 0.40 \pm .06 | 105 \pm 17 |
| Sural | 0 | 14 | 76.3 \pm 1.0 | 152 \pm 7 | 382 \pm 15 | 1.22 \pm .09 | 369 \pm 41 |
| | 100 | 15 | 63.0 \pm 1.6 | 214 \pm 10 | 400 \pm 18 | 0.93 \pm .05 | 340 \pm 27 |
| | 100 + 100 | 5 | 43.9 \pm 3.7 | 371 \pm 39 | 417 \pm 34 | 0.24 \pm .06 | 101 \pm 24 |
| | 300 | 5 | 60.1 \pm 3.0 | 252 \pm 18 | 341 \pm 29 | 0.40 \pm .12 | 122 \pm 36 |

when the final degeneration process was started ("initial") and 25 days later at autopsy ("final").

Table 3 summarizes the data used for the analysis of cell population growth. Each of the reinnervation groups is uniform for one of the variables we have investigated, preparatory period, i.e. the time between the preparatory operation and the beginning of degeneration. This variable was introduced into the analysis to allow for any progressive effects set in train by the first operation. A second variable whose effects on population growth we wish to investigate is the fibre proportion (nerve fibre content expressed as a mean percentage of the total volume of the nerve), which we take to represent the strength of stimulus. In the next column is another of these variables, the initial density of nuclear population, before degeneration has begun. In the three remaining columns in table 3 are the three measures of population growth we have employed. The first is final density of population after degeneration, which must be investigated because of the possibility that it imposes an upper limit to the population. Growth of population as a proportion of initial population is a second important measure. In previous papers (Thomas, '48; Joseph, '47, '48, '50) proportionate growth was expressed rather roughly by dividing the final population by the initial population. Because of the exponential character of cell population growth this is inadequate for the more exact analysis we wish to attempt here. The specific growth rate (\log_e of final population minus \log_e of initial population) is the appropriate measure of proportionate growth (Medawar, '40). A third measure of growth we have investigated, the increment per unit volume, is the absolute number of nuclei added during the process of degeneration regardless of the size of the initial population, and this can be put on a comparable basis for the different nerve branches by expressing it as the number of nuclei added to those originally present in a given *initial* volume of nerve.

Though a regular pattern can be discerned in table 3, no clear picture of the factors influencing the measures of growth

can be got from these mean values, because too many variables differentiate the classes. Information about the influence of these variables must be sought by multiple regression analysis. The relation of the three independent variables (fibre proportion, initial density, and preparatory period) to each of the three dependent variables expressing growth has been ana-

TABLE 4

*Partial regression coefficients. The numbers of nerves concerned are as follows: n.g.m. 29, peroneal 51, sural 39. R is the multiple correlation coefficient, and the adjusted means were calculated by analysis of covariance of the three branches. Significance at the 5% level is indicated by *; at the 1% level by ***

| INDE- PENDENT VARIABLE | PREPARATORY PERIOD | FIBRE PROPORTION | INITIAL DENSITY | R | AD- JUSTED MEAN |
|---|-----------------------|---------------------|----------------------|-------------|-----------------------|
| Final population density as dependent variable | | | | | |
| N.g.m. | $-0.65 \pm .23^{**}$ | 3.4 ± 2.0 | $0.55 \pm .54$ | $.620^{**}$ | 350 |
| Peroneal | $-0.49 \pm .12^{**}$ | 2.2 ± 1.2 | $0.49 \pm .26$ | $.571^{**}$ | 339 |
| Sural | $-0.20 \pm .15$ | -1.1 ± 1.8 | $0.10 \pm .25$ | $.234$ | 367 |
| Branches pooled | $-0.42 \pm .08^{**}$ | $2.1 \pm 0.7^{**}$ | $0.45 \pm .13^{**}$ | $.622^{**}$ | |
| Specific growth rate as dependent variable | | | | | |
| | ($\times 100$) | ($\times 100$) | ($\times 100$) | | |
| N.g.m. | $-0.46 \pm .09^{**}$ | $3.0 \pm .9^{**}$ | $-0.32 \pm .21$ | $.947^{**}$ | 1.067 |
| Peroneal | $-0.21 \pm .05^{**}$ | $1.6 \pm .5^{**}$ | $-0.25 \pm .09^{**}$ | $.947^{**}$ | 0.817 |
| Sural | $-0.18 \pm .05^{**}$ | $1.2 \pm .6$ | $-0.23 \pm .09^{*}$ | $.851^{**}$ | 0.897 |
| Increment per unit volume as dependent variable | | | | | |
| N.g.m. | $-0.75 \pm .23^{**}$ | $4.6 \pm 2.0^{*}$ | $-0.32 \pm .52$ | $.869^{**}$ | 199 |
| Peroneal | $-0.51 \pm .12^{**}$ | $3.8 \pm 1.2^{**}$ | $-0.18 \pm .26$ | $.869^{**}$ | 185 |
| Sural | $-0.67 \pm .28^{*}$ | 0.9 ± 3.3 | $-0.39 \pm .46$ | $.647^{**}$ | 288 |

lysed separately. Within each nerve branch the different times of reinnervation were pooled, but the three branches have been kept distinct. The results are set out in table 4, which shows the partial regression coefficients, with their standard errors, for the regression of each measure of growth on each independent variable. Also tabulated are the value of the multiple correlation coefficient, whose square expresses

the proportion of the variance accounted for by the three independent variables combined, and the mean of the dependent variable adjusted (by covariance analysis) so as to standardise the effects of the regression as between the different nerve branches.

On the whole the three nerve branches are similar in their partial regression coefficients, though the sural is a little different from the other two in that fibre proportion in no case has a significant effect. We will consider separately the three dependent variables representing growth.

Final population density. As table 3 shows, the final density of population is roughly the same in the different nerve branches; in the 0 day group it is remarkably the same, which confirms the conclusions of Joseph ('50). Application of the multiple regression analysis to this measure of growth, treating the nerve branches separately, shows that a significant relation holds only with preparatory period and even that fails in the sural. In the n.g.m. and peroneal, the longer the preparatory period the lower the final density. There is however no significant difference between the means (given in table 4) of the three nerve branches when they are adjusted for the differences in their values of the independent variables by analysis of covariance ($F=1.33$, d.f. 2 and 113, $P > 0.2$). Nor do the regression coefficients differ significantly as between the different nerve branches; though there is some variance in excess of chance between the different nerves in the total regression effect ($F=2.33$, d.f. 6 and 107, $0.05 > P > 0.01$), due to the divergence of the sural. It is certainly legitimate therefore to pool the results from the n.g.m. and peroneal. When this is done the partial coefficients are, for fibre proportion 2.2 ± 0.9 (significant at 2% level) for preparatory period -0.50 ± 0.10 (significant at 0.1% level) and for initial density 0.40 ± 0.19 (significant at 5% level). When the data of the three branches are pooled, all three independent variables prove to have a significant effect at the 1% level (table 4). The values of the coefficients do not represent the relative effectiveness of the three independent variables in

their control of the population density in our material, since some have a wider range of variation than others. Coefficients standardised for range of variation show that preparatory period is most important, with initial density and fibre proportion approximately equal. The multiple correlation coefficients given in table 4 for this growth measure are not high; that for the sural is not even significant. Most of the total variance is not in fact accounted for by the analysis in terms of the three variables we have used.

Specific growth rate. The means of this measure in table 3, in contrast to those of final density, seem to show a clear trend with one or both of fibre proportion and initial density. The regression coefficients of table 4 are in fact all significant, except that for fibre proportion in the sural branch and that for initial density in the n.g.m. There is a difference of borderline significance in the adjusted means ($F = 3.68$, d.f. 2 and 113, $0.05 > P > 0.02$), the n.g.m. being the exceptional nerve. There are considerable and significant differences between the branches in some of the regression coefficients. The standardised coefficients show that in our range of data all three variables are about equally important in determining the growth rate. Ninety per cent of the variance is accounted for by the regression in the n.g.m. and peroneal branch.

Increment per unit volume. The means of this measure in table 3, like those of specific growth rate, suggest a clear relationship with either or both of fibre proportion and initial density. The regression analysis shows that the significant relation (though again not for the sural) is with fibre proportion. The greater the amount of nerve fibre in a unit volume of nerve at the time when degeneration is started, the more nuclei are added to the existing population within that unit volume. All three branches show a negative relation to initial density of population, but it is significant in none; nor is it significant when all the data for the three branches are combined by pooling their sums of squares and products (partial regression coefficient $-0.22 \pm .23$; $t = 0.98$, d.f. 113, $0.4 > P > 0.3$); nor when the raw data of peroneal and n.g.m. are

pooled (partial regression coefficient $-0.29 \pm .19$; $t=1.50$, d.f. 76, $0.2 > P > 0.1$). Pooling of the raw data of all three branches produces a positive relation to initial density (the partial regression coefficient is 0.24 ± 0.17) which is also not significant ($t=1.39$, d.f. 115, $0.2 > P > 0.1$). Such pooling of all three branches is however hardly legitimate: although the partial or total regression coefficients are not significantly different between the three branches, the mean number of nuclei added in the sural branch is very significantly higher than in the other two branches when allowance is made for the effects of the regression by analysis of covariance (for the difference between the three adjusted means $F=10.6$, d.f. 2

TABLE 5

Growth rate during degeneration of nerves which had undergone no previous operation: comparison of two different age-groups of rabbits. N = number of nerves

| NERVE BRANCH | AGE GROUP | N | SPECIFIC GROWTH RATE |
|--------------|-----------|----|----------------------|
| N.g.m. | 100 | 8 | $2.71 \pm .12$ |
| | 300 | 6 | $2.63 \pm .15$ |
| Peroneal | 100 | 10 | $1.76 \pm .06$ |
| | 300 | 9 | $1.63 \pm .09$ |
| Sural | 100 | 9 | $1.28 \pm .12$ |
| | 300 | 7 | $1.10 \pm .07$ |

and 113, $P < .001$); the n.g.m. and peroneal are again very similar to each other. The standardised regression coefficients show that preparatory period and fibre proportion are almost equally effective in controlling the addition of nuclei. Only a quarter of the variance in peroneal and n.g.m. is independent of the regression.

Multiplication in relation to age of animal. The 0 day group of nerves had two sources. They came from the unoperated side of animals whose other side provided either 100 day or 300 day reinnervated nerves. Though the ages of our animals are not known, we can be sure that those providing the 300 day nerves were, at the time of the final operation, considerably older than those providing the 100 day nerves. The specific growth rate of the 0 day nerves (table 5) is lower in

the older animals. It is not significantly so in any one of the three branches, so the data from all three branches were treated together by analysis of variance. Interaction between branches and ages was not significant. Testing the variance due to age against the variance due to individuals, $t = 1.75$, d.f. 43, $0.1 > P > 0.05$. The effect is not therefore significant, though the result suggests that experiments on animals of known ages might give significant results.

DISCUSSION

Our object in this work has been to obtain information about factors influencing the growth of nuclear population in degenerating peripheral nerve. We will discuss successively the influence of the three variables that we have used to express the state of the nerve at the beginning of the degeneration process: duration of preparatory period, proportion by volume of nerve fibre, and initial density of nuclear population.

Influence of duration of preparatory period. The most effective of the three variables was the duration of the period between the preparatory operation, which established a raised cell population, and the second operation, which began the final period of degeneration. Other conditions being equal, the longer the preparatory period the less was the total amount of growth achieved. We suggest that in a nerve which has once been degenerated the cells undergo a cumulative diminution of their reactivity to the growth stimulus as a result of the prolonged presence of a cell population density largely in excess of normal. The excess may be relative to the available blood supply, which is probably not increased proportionately to the size of the new population because there is no branching of capillaries during degeneration (Abercrombie and Johnson, '46).

Influence of fibre proportion. The dissolution of the nerve fibres provides the stimulus which sets going the growth of nuclear population. In the n.g.m. and peroneal the total amount of this growth is greater the larger the initial quantity

of nerve fibre. In the sural branch the relationship of growth to fibre proportion is not significant, but we are unwilling to concede that the sural has a different mechanism of growth control from the peroneal and n.g.m. We conclude therefore that no hypothesis is likely to be right which implies that the response to fibre dissolution is merely all-or-nothing. One such hypothesis is that the amount of growth achieved is controlled, independently of the amount of nerve fibre destroyed, simply by the existence of a limiting density of population, beyond which growth is stopped. There is no doubt that, as Joseph ('50) has emphasised, a variety of different nerves have similar final densities after degeneration; the relatively poor prediction of final density by the regression on the three independent variables, that we find in our data, is an expression of these similarities. Their explanation, it seems to us, is that the volume of a degenerated nerve is to some extent an outcome of its total content of cells, and not vice versa as the hypothesis of a limiting density supposes.

Influence of initial density. Though we have excluded density of population as the sole controlling agent, it may still have some influence on the amount of growth. The data show in fact some significant correlations of initial density with two of the measures of growth, when due allowance has been made for differences of preparatory period and fibre proportion. Initial density is positively correlated with final density when the data from the three different branches are pooled; and it is negatively correlated with specific growth rate, though not significantly in the case of the n.g.m.

These correlations allow us to eliminate the hypothesis that control is exercised simply by a relation between concentration of stimulant and specific growth rate (i.e., mitotic rate), since initial density should in that case be irrelevant. It is not however so easy to interpret unequivocally the curious pattern of relations that is found. No method of treating the data will extract a significant correlation between initial density and *increment* of nuclei per unit volume. Our

sample of nerves was a large and varied one; and though it is possible that a larger sample still might establish a significant relation, we are at present justified in the supposition that there is no correlation or only a very feeble negative one between initial density and increment of cells per unit volume. If we accept this assumption, we require a hypothesis by which, preparatory period being held constant, the number of new cells appearing in a unit volume is substantially determined by the amount of stimulant released in that volume, regardless of the number of cells initially there. Two possible hypotheses may be suggested.

1. The stimulant is not significantly inactivated by the cells present, except during the actual formation of each new cell: i.e. appreciable inactivation occurs only during the process of mitosis. The correlations of initial density with specific growth rate and with final density would in this case be purely formal: a given increment will obviously entail a higher growth rate and a lower final *population* the smaller the initial population to which it is applied; the lower final *density* actually observed will follow only if swelling of the nerve during degeneration is not related to initial density, but we have actually found that it is not related.

A specific form of this hypothesis is the suggestion of Joseph ('48, '50) that the free space created by fibre degeneration is the stimulus to mitosis; it would be reduced in amount by each mitosis until it becomes zero. The theory can also be put in chemical terms by supposing that a substance is released by fibre degeneration, its presence in the nerve stimulates mitosis, and significant consumption occurs only during each cell division.

2. The data can also be adequately accounted for by supposing that resting cells inactivate the stimulant; that the total amount of stimulant available is distributed amongst the whole cell population whatever the latter's size; and that the amount of stimulant per cell determines the mitotic rate. When put in terms of increment per unit volume, the high rate of cell-division associated with the application of a

given stimulus to few cells in a unit volume must be assumed to be counterbalanced by the small number of cells available there to react, and conversely with many cells per unit volume; the two effects of population density cancelling out.

Difference between nerve branches. Nerve branch (n.g.m. peroneal or sural) is a further variable distinguished in our data. In general the correlations of growth in the three branches are fairly similar. It is to be expected however that there would be some differences in growth not accountable by our other variables; and though the n.g.m. and peroneal are notably alike, the sural is rather apart from these two. Indeed the hypotheses that have emerged from our data are really based on n.g.m. and peroneal only. The sural results do not contradict these hypotheses and most of the relations between variables in the sural are in fact similar to those of the other two branches. The difference is that most of them are not statistically significant. The failure to obtain any significant correlations with final density of population in this branch is doubtless due to its exceptional degree of swelling during degeneration. More difficult to account for is the absence of a relation between fibre proportion and any of the measures of growth. Perhaps the sural's high content of unmyelinated fibres, not included in our estimates of fibre proportion, is responsible.

A comment is necessary on an apparent discrepancy between the basis of Joseph's discussion ('50) of his hypothesis that liberated space stimulates mitosis, and our results. Joseph based his theory on the different amount of nuclear multiplication in various normal nerves with different fibre spectra, including the n.g.m., peroneal and sural; he supposed that nerves with larger fibres, which have a higher multiplication, have a larger total volume of nerve fibre. We find however that these three nerves do not differ appreciably in their fibre proportions, that is to say in the amount of space which could be made available by degeneration. They do not therefore provide evidence for the space theory. The difference in the amount of multiplication of their cells is

actually to be referred to the addition of rather similar numbers of cells to very different initial numbers. The same explanation probably holds for the remarkably linear relation between mean fibre diameter and multiplication of cell population, found by Thomas ('48). An inverse relation between mean fibre diameter and initial population of Schwann cells is readily explicable. Since there is one Schwann nucleus per internode in normal nerves, and a positive relation between fibre diameter and internode length (Vizoso and Young, '48), density of Schwann cells will be inversely related to mean fibre diameter. From the data of Abercrombie and Johnson ('46) and Thomas ('48) it can be calculated that there is a similar relation for endoneurial cells, the number per mm^3 in the large-fibre n.g.m. being 15,000, in the intermediate peroneal 21,000, and in the small-fibred sural roughly 40,000.

SUMMARY

The main purpose of this work was to assess the importance of intensity of stimulation and of density of cell population in controlling the multiplication of cells during Wallerian degeneration in rabbit sciatic nerves. The quantity of nerve fibre present in the nerve at the beginning of degeneration was taken to represent the quantity of stimulant. Nerves were first prepared by crushing them and allowing them to become reinnervated. By varying the length of time of reinnervation and by repeated crushing, the quantity of nerve fibre and the initial population can be varied over a considerable range. Analysis by multiple regression of the growth in nuclear population that occurs during a subsequent period of degeneration shows that the amount of growth depends on the amount of stimulant; and that at a given level of stimulation a certain number of new cells is added within a given volume of nerve regardless of the number of cells already present there. Two plausible kinds of stimulus-response mechanism having this property are suggested. In addition the amount of growth is strongly depressed by increasing the time between

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the preparatory operation, which establishes a high population of cells in the nerve, and the final Wallerian degeneration.

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ONTOGENETIC CHANGES IN THE FREEZING POINT AND SODIUM AND POTASSIUM CONTENT OF THE SUBGERMINAL FLUID AND BLOOD PLASMA OF THE CHICK EMBRYO ¹

EVELYN HOWARD

*Department of Physiology, The Johns Hopkins University,
School of Medicine, Baltimore 5, Maryland*

ONE FIGURE

The importance to morphogenesis of the osmolarity of the environment of the embryonic chick during the first two days of incubation has been demonstrated by studies of chick blastoderms in tissue culture (Howard, '53). The observations indicated the desirability of obtaining information regarding the course of the transition from the low osmolarity of the unincubated egg, equivalent to 123 m-eq. of NaCl, to that of adult chicken plasma, which corresponds to about 160 m-eq. of NaCl (Howard, '33, '44). The present paper reports measurements of the freezing point depression of subgerminal and amniotic fluids and chick embryo blood, obtained at suc-

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Preliminary reports of part of this work were presented at the Federation meetings in March, 1953, and at the XIX International Physiological Congress in September, 1953.

cessive stages of development.² Freezing point studies have been supplemented by observations on the sodium and potassium content of the various fluids, partly in order to see how closely the measurements were correlated, but also because of the interest which attaches to potassium disposition in the avian egg.

The supply of K carried by the hen's egg is known to result in K values of the order of 40 m-eq. in the white, and somewhat more in the yolk (Needham, '31b). These amounts are well outside the range that would be tolerated in adult plasma, serum K in the adult fowl being 2.6 to 4.7 m-eq. (Hunter, '51), not in excess of the normal mammalian range. Cardiac arrest occurs in dogs at 14 to 16 m-eq. of plasma K (Winkler et al., '38), and the chick embryo heart *in vitro* reacts to changes in K concentrations similarly to the adult heart (Lewis, '29). Presumably the embryonic tissues at some time become protected from the high K content of the unincubated egg fluids, but when such a process becomes manifest, and how it is mediated, has not, to the author's knowledge, been examined.

Evidence that the embryo does not metabolize the yolk to any great degree before the second week of incubation (Needham, '31b) suggests that the subgerminal fluid is derived chiefly from the egg white. This view finds support in observations of Romanoff ('43), who finds that during the first five days of incubation, when the subgerminal fluid or "liquefied yolk" is increasing rapidly in volume, the egg white is decreasing at a similar rate, whereas the yolk is practically unchanged in volume. Furthermore, New ('56) has observed that chick blastoderms in culture accumulate fluid at the ventral surface. Comparisons of K/Na ratios in subgerminal fluid and egg white, which will be presented below, suggest

² The literature contains some observations (Bialaszewicz, '12, and Kamei, '27) which agree in indicating that chick amniotic fluid is distinctly hypotonic to hen blood. However, the values for both blood and amniotic fluid are somewhat greater than found in the present study, presumably because of the use of excessive supercooling in the freezing point determinations. Korr ('39) found adult fowl blood vapour pressure to be equal to 158 to 161 m-eq. NaCl, using Hill's thermoelectric method.

that there may be an active transfer of Na from egg white to subgerminal fluid, mediated by the blastoderm. The yolk sac, or non-embryonic portion of the blastoderm, is known to be a metabolically active tissue: it produces acid phosphatase at a high rate (Mezger, '49), and Needham ('30) states that "there can be no doubt that the yolk sac has important metabolic functions to perform . . . for xanthine oxidase and ovomucoidase are to be found in it long before they make their appearance in the embryo itself."

Previous studies on the functional maturation of the mesonephros are of interest in relation to other aspects of the observations to be presented. The allantois becomes vesicular at stage 20, and water is passed through the mesonephros against pressure soon after this (Boyden, '24). Fiske and Boyden ('26) reported the first unequivocal evidence of an increased uric acid concentration in the chick allantoic fluid in the 5 day embryo, and Chambers and Kempton ('33) reported that cultures of mesonephric tubules from chick embryos first began to show their characteristic ability to concentrate phenol red at 4.5 days. Thus, from this time on the mesonephros could begin to be a factor in the regulation of the osmolar level of the blood. Chambers and Kempton further found that from the twelfth to the eighteenth day there is a slow diminution in the ability of the mesonephric tubules to accumulate phenol red, and after the eighteenth day this decline in secretory activity becomes very marked. "On the eighteenth day the concentration of the dye by the tubules is considerable, but when the tubules of 20 day embryos are studied there is a striking difference. The cells of the tubules . . . are highly granular and almost opaque, many of the tubules being composed of cells which appear dead. A slight amount of concentration may, however, occur in a few of the more healthy tubules." In a 19 day chick the mesonephros has regressed considerably, while the metanephros has attained conspicuous proportions. The metanephros has been shown by Gersh ('37) to be capable of functioning concurrently with the mesonephros in the chick, on the basis of phenol red

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concentrating ability. In 11 day embryos, Gersh observed the first sign of phenol red accumulation in one or two metanephric nephrons in each lobule. Thereafter the number of active metanephric tubules increased rather gradually.

The present observations show a gradual rise in the osmolar concentration of the embryonic milieu during the progress of development, with a rather sharp transition of the plasma to the adult value at the time of hatching, when the mesonephros ceases to function.

METHODS

Chemical: Na and K were determined by means of a Li internal standard flame photometer. Unknowns were read between two readings of standard solutions. Values were reproducible to within 1% of the molarity being measured. Mutual interference effects were tested and found negligible except at plasma concentrations, where the K values were about 6% too high. This correction was not applied in the tables, since it would not materially influence the matters under examination. Subgerminal fluid and amniotic fluid were centrifuged to remove any possible cellular debris, and diluted, in the manner usual for plasma, to a suitable concentration range for the photometer. Egg white was in some cases "wet ashed" by liquefying with HNO_3 and removing excess nitrates with formic acid, according to the procedure used by Walker and Wilde ('52). Alternatively, samples were diluted with 6 volumes of water and homogenized. Aliquots of the homogenate were further diluted 1:10 or more for photometer reading. Determinations on these homogenates were satisfactorily reproducible and concordant with the "wet ashed" samples. Nitric acid liquefaction was unsuitable for egg yolk because of its high fat content. The yolk was diluted gradually with 200 volumes of LiCl solution, to a final Li concentration of 50 parts per million, which produced a uniform dispersion which gave reproducible readings in the photometer.

Freezing point determinations were performed using the classical Beckmann method, as modified by Johlin ('31) for

samples of 2 ml. The fluids reported on in this paper all showed a fairly prompt rise of temperature, following seeding of the supercooled material with a minute ice crystal, in the classical manner characteristic of dilute solutions. In the present work the procedure was to cool the material 0.15 to 0.20°C below the expected freezing point, seed with a nichrome ring cooled on dry ice, and thereafter maintain the bath temperature to within 0.10 to 0.03°C below the expected freezing point, while following the temperature for about twenty minutes, reading the thermometer every thirty seconds while maintaining a uniform rate of stirring. Values given are the mean of temperature readings after the temperature had remained constant to within a few thousandths of a degree for 15 or 20 minutes. Determinations of the freezing point of water were made on each day. Determinations of the freezing point of a standard NaCl solution checked the theoretical to within 0.002°C. The overall scatter of the data is indicated in the figure and tables.

Biological: Blood was drawn from chick embryos 11 days of age and over by means of a fine glass needle inserted in a chorio-allantoic vein under 15 × magnification. A window was made in the shell, and the shell membrane rendered less opaque by painting it with mineral oil so that an appropriate vein could be visualized. As a precaution against contamination of the sample with allantoic fluid, before inserting the needle in the vein the underlying allantoic fluid was drawn off and air injected so that the vessel from which the blood was drawn was separated from the underlying fluid by several mm of air. Blood samples were chilled when obtained, and kept cool during the time required to draw blood from 6 to 8 embryos, after which the freezing point was immediately determined. Blood from embryos at 11 and 12 days of age did not clot, but with embryos 16 days and over, heparin was used as an anti-coagulant. After chicks had hatched, blood was obtained by heart puncture, usually under light nembutal anesthesia. Blood from hatched chicks was immediately centrifuged under oil. Before hatching, the viscosity of the blood is

so low that whole blood is satisfactory for the freezing point determination, but after hatching plasma is preferable.

To obtain amniotic fluid, a small window was made in the shell and the allantois opened, so that on subsequently opening the egg into a Petri dish, the chorio-allantois could easily be separated from the amnion without rupturing the latter. Surrounding fluids were removed, the clean surface of the amnion punctured with a fine needle, and the fluid withdrawn by gentle suction. To obtain subgerminal fluid, a small area of the shell and the shell membrane were removed directly over the embryo, and a fine needle inserted into the space under the embryo.

Eggs were incubated at $38.0 \pm 0.5^{\circ}\text{C}$ and at 50% humidity $\pm 5\%$. Hamburger and Hamilton ('51) stage numbers have been used to indicate the developmental age of the embryos. After hatching, the general health of the chicks was evidenced by the fact that they ate and drank readily and more than doubled their hatching weight in two weeks.

OBSERVATIONS

In the unincubated egg the subgerminal fluid is represented by a thin film of clear fluid between the central zona pellucida and the underlying yolk. During incubation the volume of the subgerminal fluid increases rather rapidly, so that sufficient quantities for analysis could be obtained by stage 14 to 15 (22 to 27 somites).

The data are summarized in tables 1 and 2. Na and K in unincubated egg white were reasonably constant in different samples, as was the Na in egg yolk, but the K in different samples of egg yolk was more subject to variation, for reasons which are not apparent. It can be seen that the Na of the subgerminal fluid was rather variable during day 2, thereafter, except for day 11, values were more uniform. The magnitude and wide scatter of the K figures on day 11 presumably reflects the greater incorporation of yolk materials by this stage. As development proceeds, the subgerminal fluid shows some decline in Na, with a considerable increase in K.

The sum of the two rises slightly after the second day and then shows little change before day 11. The contribution of Na and K salts to the freezing point depression has been calculated, using the factor for NaCl at this molarity, where equivalent per liter of water $\times 3.47$ equals the freezing point depression. It will be seen that the observed freezing point depressions of the plasma and amniotic fluid are approximated

TABLE 1

Na and K contents and freezing points of subgerminal fluid of chick embryos, compared with yolk and white of unincubated eggs

| AGE | STAGE | Na | K | WATER | Na + K | FREEZING POINT | |
|-------------|-------|-----------------|-----------------|----------|------------------------------|----------------|-----------|
| | | | | | | Calc. | Observed |
| <i>days</i> | | <i>m-eq./L</i> | <i>m-eq./L</i> | <i>%</i> | <i>m-eq./LH₂O</i> | <i>°C</i> | <i>°C</i> |
| 0 | White | 85.9w \pm 3.4 | 42.3w \pm 1.4 | 88 | 128.2 | —0.449 | —0.424 |
| 0 | Yolk | 38.9w \pm 2.1 | 56–89w | 48 | ? | ? | .420 |
| 2 | 14–15 | 103.4 \pm 7.3 | 12.7 \pm 0.5 | 97 | 119.8 | .416 | — |
| 2.5 | 16–17 | 98.6 \pm 5.6 | 15.7 \pm 2.0 | 97 | 117.8 | .409 | — .469 |
| 4 | 20–24 | 106.1 \pm 1.1 | 20.4 \pm 1.1 | 97 | 130.4 | .452 | .472 |
| 6 | 29 | 94.7 \pm 1.4 | 24.8 \pm 1.1 | 96 | 124.5 | .432 | .488 |
| 8 | 33–34 | 88.9 \pm 1.7 | 34.0 \pm 0.9 | 95 | 129.4 | .449 | — |
| 11 | 37 | 61.4 \pm 3.0 | 42.9 \pm 33.7 | 87 | 120.0 | — | .505 |

Freezing points were calculated as the contribution of Na, K and univalent anions, using the constant for NaCl at this molarity, where equivalent per liter water times 3.47 equals the freezing point expected. Subscript *w* indicates per liter of water. Stage numbers according to Hamburger and Hamilton ('51). Figures for Na and K are followed by range among at least 3 samples. The sample scatter is not analytical beyond 2 per cent. The standard deviation of freezing point determinations from the respective means was 0.007°C for subgerminal fluids. Water content was not determined on all samples: see text.

rather closely by the contributions of the Na and K salts evaluated in this way. The correspondence is less close for the subgerminal fluid than for the plasma and amniotic fluids. Although the figures are usually for fluids from different specimens, the discrepancies suggest the presence of appreciable amounts of a variety of solutes in the subgerminal fluid. In the case of the plasma, a notable discrepancy occurs at the time of hatching, when the freezing point had achieved essentially its final value, whereas within 1 to 2 hours after hatching

the Na was intermediate between the embryonic and the final level. Although freezing points were not done on the same specimens as the analyses, the findings suggest that the final ionic adjustment was somewhat slower than the adjustment of the mol fraction of water. This might be accounted for by accumulation of lactate in the first hours following the efforts of hatching and assumption of air breathing.

The plasma and amniotic fluid K figures ranged between 3.0 and 4.9 m-eq., the maximum being at the time of hatching. There is no indication from these figures that chick embryo tissues are ever exposed to unusually high levels of K. Fur-

TABLE 2
*Na and K contents and freezing points of amniotic fluid and
blood of chick embryos*

| AGE | Na | K | WATER | Na + K | FREEZING POINT | |
|----------------|----------------|----------------|----------|------------------------------|----------------|-----------|
| | | | | | Calc. | Observed |
| <i>days</i> | <i>m-eq./L</i> | <i>m-eq./L</i> | <i>%</i> | <i>m-eq./LH₂O</i> | <i>°C</i> | <i>°C</i> |
| Amniotic fluid | | | | | | |
| 6 | 136.3 | 3.0 | 99.0 | 140.7 | -0.488 | — |
| 8 | 136.4 | 3.3 | 99.0 | 141.1 | -0.490 | -0.493 |
| 11-12 | 142.2 | 3.9 | 99.0 | 147.6 | -0.512 | -0.503 |
| Plasma | | | | | | |
| 11 | 137.7 | 4.5 | 97.2 | 146.3 | -0.508 | — |
| 12 | 132.6 | 4.2 | 97.2 | 140.7 | -0.488 | -0.504 |
| 16 | — | — | — | — | — | -0.516 |
| 18 | 132.7 | 3.7 | 95.7 | 142.5 | -0.494 | -0.526 |
| 0 P.h. | 140.5 | 4.9 | 93.8 | 155.0 | -0.538 | -0.572 |
| 2-20 P.h. | 149.6 | 3.6 | 93.8 | 163.3 | -0.567 | -0.577 |

Freezing points calculated as in table 1. P.h., post hatching. Figures for water contents of amniotic fluid from Kamei ('27) and for bloods from Schechtman ('52). Plasma was used except for the freezing point determinations in embryos, where whole blood was employed. Sodium figures are means of at least three separate samples. Individual "samples" were pools from 2 to 8 embryos. The standard deviations of all sodium measurements from the means of the respective groups were: for amniotic fluids and embryonic plasmas, 1.4 m-eq., for hatched plasmas, 2.7 m-eq. Plasma potassiums were usually single determinations, amniotic potassiums were done in triplicate: standard deviations for all potassiums were 0.2 m-eq. The standard deviation from the respective means for all freezing points was 0.006°C.

thermore, the subgerminal fluid K at its lowest observed point, 12.7 m-eq., beneath embryos of 22 to 27 somites, although still considerably higher than any observed plasma K, was much lower than the K in either the white or yolk. At these early stages the subgerminal fluid thus prevents the embryo from having vascular contact with the high K characteristic of the unincubated yolk or white. The subgerminal K rises gradually as development progresses, but from stage 20 on the mesonephros is presumably capable of regulating embryonic plasma K. During days 6 to 11, amniotic fluid and plasma K show no indication of being altered by the high K concentrations existing in the subgerminal fluid. Blood was not obtained early enough to determine directly whether the embryo is capable of maintaining the embryonic plasma K at 5 m-eq. during the second day when the subgerminal fluid K is 12.7, although such a degree of regulation would seem not unreasonable.

It is at first surprising that the underlying yolk does not liberate enough K to raise the subgerminal K above the observed value for the early stages. The yolk appears to be a separate layer under the subgerminal fluid, but no membrane barrier separates them. It is apparent that K does not diffuse out of the yolk at a rate great enough to permit a concentration equilibrium to be achieved during the first few days of incubation. In this connection, it is interesting to note that conductivity measurements of Maurice ('52) have indicated that unincubated yolk has a diffusional resistance about 200 times that of 1% NaCl. Philip W. Davies and the author have confirmed this observation with respect to the order of magnitude of the conductivity of mechanically undisturbed yolk.

A few observations on the changes in the egg white during the course of development were made. As the blastoderm grows down over the yolk surface, the white is known to recede, so that the white comes to be in contact with the blastoderm only at the peripheral portions of the latter. Hence the white is not necessarily in diffusion equilibrium with the subgerminal fluid. The white was sampled for analysis by insert-

ing a large needle through a window at the end of the egg, directed downward toward the bottom surface of the shell, and withdrawing a sample of the more fluid egg white. In the white at 6 days (stage 29), the mean values were: K 61 and Na 54 m-eq. per liter. The K/Na ratios were, in three cases, 0.91, 1.47 and 1.09, in contrast to the subgerminal fluid at stage 29, which had a K/Na ratio of 0.26, and the unincubated egg white, which had a ratio of 0.49. These ratios are in accordance with what would be expected if the blastoderm was actively transferring Na from the white to the subgerminal fluid, so that, as the volume of subgerminal fluid is increasing, Na is entering against a concentration gradient, while K accumulates in the white. If Na is actively transferred, water would presumably tend to follow Na osmotically, so that one need not postulate active transfer of water to account for the accumulation of fluid in the subgerminal region.

The total solids in the subgerminal fluid probably vary somewhat depending on how much the underlying yolk layer has been mechanically disturbed, or subjected to convection currents. For the purposes of this study, the eggs were not turned during incubation. In five eggs with embryos at stage 20, the fluids were all translucent but not clear; two were similar to egg white in color and had 2.5 and 3.3% solids; three others were yellow and had 4.4, 4.6 and 6.0% solids. At 6 days, solids were 2.9, 3.2 and 5.1%; at eight days, 4.7, 4.8 and 5.7%. Even at 11 days, when yolk breakdown is under way on a large scale, it has been possible to draw off a small amount of fluid of 3.1% solids from the region adjacent to the yolk sac, although the aliquot analyzed had 13% solids. Possibly the fluid secreted by the yolk sac has a total solid content of the order of 3% or less, and this is increased by admixture from the underlying yolk: the degree of mixing increasing considerably as metabolic utilization of the yolk becomes appreciable, and when active movements of the amnion begin.

The freezing point data presented in the tables are summarized graphically in figure 1. The freezing point depression of subgerminal fluid obtained from beneath embryos of 25 to

32 somites, the earliest stage from which it could be obtained in convenient quantities, was -0.47°C , a value appreciably greater than that of unincubated egg white, -0.42° . If some degree of increased osmolality in the subgerminal fluid follows the initiation of development it might in part account for the fact that by the 22 somite stage the white has been observed to be entirely absorbed from a considerable part of the central area of the dorsal surface of the blastoderm. One result of this absorption of the overlying white is that the vascular area of the blastoderm becomes closely applied to the shell membrane without any intervening fluid layer, and becomes an effective oxygenating surface at this stage. Differences in the oxygenation of the blood in arteries and veins

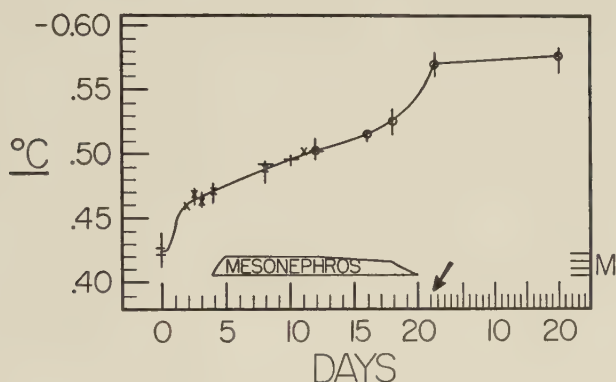


Fig. 1 Ontogenetic changes in the freezing point depression of various embryonic milieus in the chick. Abscissae, age in days of incubation to day 21, thereafter, days after hatching. Ordinates, freezing point depression in $^{\circ}\text{C}$. The line is drawn freehand. Bars indicate scatter of observations for a given age, means being indicated by the following symbols for different fluids: \equiv , unincubated egg white; \times , subgerminal fluids; $-$, amniotic fluids; 0 , bloods. The duration of functional activity of the mesonephros is indicated on the basis of observations of others discussed in the text: the ordinates of the mesonephros scale (M) indicate roughly the intensity of the functional indices. The metanephros begins to show phenol red accumulation in a few nephrons at 11 days, active tubules increasing gradually thereafter. The plateau after hatching is within the range noted for the freezing point depression of the blood of a small group of adult laying hens (Howard, '33). Mean values for adult fowl blood calculated from vapor pressure data reported by Korr ('39) were -0.554°C and by Aldred ('40) -0.608°C .

is clearly visible, under $15\times$ magnification, in blood coming from an undisturbed area of the vitelline circulation, prior to the development of the allantois, if one makes a small window in the shell. However, this visible difference disappears rather quickly if the vascular area is allowed to dry after the shell membrane has been removed.

When development is initiated, diffusion gradients are presumably set up between the germinal disc and its surroundings, but when blood circulation becomes established, at about the 16 somite stage, one might expect that the blood stream and the subgerminal lake would tend to approach osmotic equilibrium. There is no evidence that the pronephros is ever functional in the chick. Embryos of 30 to 40 somites (stages 17 to 19) with the amnion not yet closed and the mesonephros not yet functional, before the allantois is vesicular, are serviced by an oval vascular area of 10 to 15 mm radius. Such embryos weigh on the order of 20 mg or less, and are in vascular contact by means of the vitelline circulation with a volume of two to four ml of subgerminal fluid. There seems to be no reason to suppose that such embryos would maintain a plasma osmolarity different from that of the subgerminal fluid. At stage 20, when the allantois is vesicular and about the size of the midbrain, the subgerminal fluid freezing point was not significantly different from the preceding stages.

After six days of incubation, at stage 29, the amnion has become completely closed and the mesonephros is well established in functional activity. In this group the subgerminal fluid was found to be slightly more concentrated. The amniotic fluid could be obtained at this stage, and its freezing point (see table 2) did not differ appreciably from that of the subgerminal fluid. This equivalence of the amniotic and subgerminal fluids tends to support the inference that the embryonic blood, as the functional intermediary between the two, has a similar total osmolarity, and the correspondence between blood and amniotic fluids was directly demonstrated in embryos of 12 days of age, when blood could be obtained in sufficient quantity for study.

The freezing point depression of amniotic fluid increased slightly between 6 and 12 days. During the stages studied here, the freezing point of the amniotic fluid corresponds quite closely with that expected from the conductivity measurements of Walker ('43), on the assumption that the amniotic fluid approximates a uniunivalent salt solution. At 12 days of age, under the present incubation conditions, the amniotic cavity had not opened into the egg white reservoir: later on this occurs and the conductivity of the amniotic fluid drops to a level hypotonic to plasma. However, from the time of closure of the amnion to the twelfth day, plasma and amniotic fluid may be expected to have essentially the same freezing point. As may be seen in the figure, the freezing point of the embryonic blood changes little between 12 and 18 days of incubation, but there appears an abrupt discontinuity at hatching, with an immediate shift to the adult level. The period of presumed activity of the mesonephros has been indicated in figure 1 on the basis of studies of others mentioned above. It can be seen that during the period at which the mesonephros is functional, the freezing point depression of the various milieux increase at a decreasing rate, and that an abrupt rise to a plateau occurs at the time of cessation of function by the mesonephros.

COMMENTS

The observations suggest that active secretion by the blastoderm participates in the formation of a subgerminal fluid with a K concentration lower than that of the unincubated egg fluids, so that, at least by stage 15, the embryo is shielded from vascular contact with the initially high egg K. There is no evidence, at the stages at which blood was examined, that blood K is higher than the usual adult range. However, these studies leave open the interesting question of the effective extracellular K at very early developmental periods.

It is pertinent to consider how much the overall loss of water by the egg during incubation might contribute to the observed increase in osmolality. The actual amount of water lost depends on the incubator humidity. At 65 to 70% hu-

midity, considered optimal for viability, Murray ('25) observed a water loss of 9.5% of the initial water content. This was accompanied by the metabolic loss of 10.3% of total solids, so that the overall ratio of solids to water changed remarkably little. The loss of 9.5% water can be compared with the 26% increase in freezing point depression, i.e., from -0.42 to $.53^{\circ}\text{C}$ before hatching. Thus, it can be seen that the ontogenetic shift in osmolarity permits the egg to vaporize a considerable amount of its initial water without infringing on its final osmolarity.

There is evidence of some degree of ontogenetic shift in osmolarity in other vertebrates. Embryonic development in the frog takes place in an internal milieu which during gastrulation is some 33% more dilute than that of the adult frog (Krogh et al., '38). Similarly, the trout egg has been shown to take up water after it is shed (16% of its initial weight, Manery and Irving, '35). Birds' eggs which have been examined include the duck *Anas*, and the goose, *Anser*, whose eggs were found by Atkins ('09) to have the same hypotonicity to the adult blood as he reported for the hen's egg. The question arises as to whether these dilutions of the embryonic milieu have a specific facilitory effect on embryonic development, possibly for phylogenetic reasons, or whether they are simply circumstances to which the embryos have become adapted. In the case of the trout and the frog, the circumstance of being extruded into fresh water is associated with an initial osmotic uptake of water, which is terminated by the surface coating becoming highly impermeable to water (Krogh, et al., '38, and Krogh, '39). However, even in mammalian embryos, there is a little evidence that the ionic strength of certain fluids is somewhat less than that of maternal plasma: namely, the Na plus K of the fluid from the interior of the 7 day unimplanted rabbit blastocyst is 14% below that of maternal serum (Lewis and Lutwak-Mann, '54), and McKay et al., ('55) have reported some preliminary figures on chorionic and amniotic fluids from 6 to 13 week

human embryos in which the Na values are less than in maternal serum.

Is it conceivable that the ontogenetic shift in osmolality has a parallel in phylogeny? It is well known that adult frog blood has a lower osmolality than bird and mammalian bloods, but the question of whether or not this is a reflection of a phylogenetic trend has been obscured by the rather wide spread of the data regarding the blood of the fishes. However, on the basis of considerations summarized by Romer and Brandon ('35), the early vertebrates may be believed to have evolved in fresh water. Hence in attempting to evaluate the osmolality of the primitive vertebrates it may be necessary to exclude not only the marine fishes, in which various forms of adaptation to the marine environment appear to have taken place subsequent to the presumed evolution in fresh water (Smith, '32), but it may be necessary to exclude also the fresh water telosts, since they "have not been, in all probability, continuous residents in fresh water since the early days of fish history: between that time and the present there intervened a long marine phase" (Romer, '55). This leaves a rather small group of fresh water vertebrates which may be supposed either to have been continuous residents in fresh water, or not to have made the adaptation to marine life which the teleosts did. Data on such forms is assembled from the literature in table 3, where it is contrasted with data on examples of the more thoroughly studied birds and mammals. The data suggest that the osmolar level of -0.43 to $.46^{\circ}\text{C}$ is one which the early vertebrate stock found to be desirable for some reason, either historical or intrinsic.³ This primitive vertebrate osmolality is not one which is rigidly adhered to, but rather an osmolality to which certain organisms tend to

³ Some terrestrial invertebrates show a tendency to adjust their osmolality toward a level similar to the fresh water vertebrates: thus, the plasma of the earthworm *Pheretima posthuma* has been reported to have a mean freezing point of 0.45° (Bahl, '45); the blood of the snail, *Helix pomatia*, $.37$ to $.43^{\circ}$ when hibernating, $.30$ to $.40^{\circ}$ when activated by moisture (Duval, '30; Kamada, '33); and the blood of African snail, *Achatina fulica*, $-0.46^{\circ} \pm .01$ (A. W. Martin, unpublished).

return after having been displaced by circumstances of varying hydration. Particularly striking are the returns following estivation in the lung fish; following embryonic dilution, as the maturing tadpole climbs back; and following somatic divergence, as in its utilization by the birds in the "private ponds" which they arrange for their embryos.

It is of interest that in ontogeny as well as, apparently, in phylogeny the shift in osmolarity is associated with the replacement of the regulating equipment, namely the transition

TABLE 3
*Freezing point depressions of the bloods of certain fresh water and
terrestrial vertebrates*

| CLASS | ANIMAL AND AUTHOR | FREEZING POINT °C |
|--------------|--|-------------------------|
| Agatha | Lamprey, <i>Petromyzon fluviatilis</i> (Galloway, '33) | — 0.46 |
| Osteichthyes | Lung fish, <i>Protopterus aethiopicus</i> (Smith, '30) | .44 |
| Amphibia | Frog, <i>Rana esculenta</i> (Hill and Kupalov, '30) | .43 |
| Reptilia | Turtle, <i>Emys europea</i> (Botazzi, '08) | .46 |
| | Tortoise (Aldred, '40) | .57 |
| Aves | Duck, goose (Atkins, '09) | .55 — .57 |
| | Chicken, pigeon (Howard, '33; Korr, '39; Aldred, '40) | .55 — .61 |
| Mammalia | Human (Gram, '24), Dog (Ball, '30), Horse (Aldred, '40) | .55 — .56 |

from the mesonephros to the metanephros. The situation suggests that the renal adjustments to the different osmolar levels may be of such a fundamental nature that it was found advantageous to develop different kidneys to cope with the different ionic strengths which are involved. What rearrangement in the internal physiology of the cells of other tissues may be associated with the ionic strength changes is less apparent. Various biochemical changes during ontogeny have been reviewed by Wald ('52). Ontogenetic changes in the types of circulating erythrocytes have been described (Dawson, '36) and possibly many cellular alterations during de-

velopment may be influenced to some extent by the observed modifications in the ionic environment.

The wide range of salinities to which modern forms have become adapted has recently been emphasized, e.g., by Beadle ('43) and by Wald ('52). The view that the known difference between the osmolality of the more primitive fresh water vertebrates, on the one hand, and the birds and mammals, on the other hand, is a fundamental difference of phylogenetic significance, rather than simply a presently existing difference of adaptation, is necessarily tentative. However, the fact that the chick embryo starts at the primitive vertebrate level appears to be a point in favor of the possibility that the present lower fresh water vertebrate level was in reality characteristic of the stock prior to the time when the characteristic avian characters appeared.

SUMMARY

Measurements of the freezing point depression of the subgerminal fluid, amniotic fluid and blood of chick embryos describe the course of an ontogenetic shift between an initial osmolality characteristic of the blood of amphibia and other more primitive fresh water vertebrates, to the osmolality characteristic of mature birds and mammals. There is a gradual shift toward higher osmolality, and a final discontinuity at hatching when the mature level is achieved, as the mesonephros ceases functioning.

The ontogenetic shift in osmolality may have survival value for a terrestrial egg inasmuch as it permits the egg to vaporize a considerable amount of its initial water without infringing on its final osmolality.

The changes in the freezing point depression are closely paralleled by changes in the Na plus K content. The K in plasma and amniotic fluid remained at 3 to 5 m-eq. At two days of incubation, subgerminal fluid K was 13 m-eq., hence the early subgerminal fluid prevents the vitelline vascular bed from being directly exposed to the high K concentrations present in the unincubated egg fluids.

Comparison of K/Na ratios and freezing point depressions in subgerminal fluid and egg white suggest that there may be an active transfer of Na from egg white to subgerminal fluid, mediated by the blastoderm, during the earlier stages of incubation. Water may follow Na osmotically, thus contributing to the characteristically rapid initial increase in the volume of the subgerminal fluid. In later stages, as the yolk gradually breaks down, a considerable rise in subgerminal fluid K occurs, but by this time the mesonephros is presumably able to regulate plasma K levels.

The early subgerminal fluid differs from both yolk and white in having much less solids than either, lower K, a pH closer to white than to yolk (Shklyar, '37) and a freezing point depression somewhat greater than that of the unincubated egg.

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BIOCHEMISTRY OF FILAMENTOUS FUNGI

V. ENDOGENOUS RESPIRATION DURING CONCURRENT METABOLISM OF EXOGENOUS SUBSTRATES¹

H. J. BLUMENTHAL,² HENRY KOFFLER AND E. C. HEATH³

*Laboratories of Bacteriology, Department of Biological Sciences,
Purdue University, West Lafayette, Indiana*

INTRODUCTION

Most cells utilize cellular materials in the absence of exogenous substrates. In the filamentous fungi the rate of the endogenous respiration usually is high as compared to the respiration in the presence of exogenous compounds. Quantitative manometric data, therefore, often are obscured by the uncertainty as to what happens to the endogenous metabolism of these organisms during the simultaneous utilization of externally furnished substrates.

Barker ('36), working with *Prototheca zopfii*, was the first to introduce a manometric method by which the total respiration of organisms can be corrected for their endogenous respiration. This method is not entirely reliable, as will be brought out later in this paper. Another approach makes use of C¹⁴-labeled cells (Burris, '49; Reiner, Gest, and Kamen, '49); the C¹⁴ released from labeled cells is assumed to re-

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²Predoctoral Fellow of the U. S. Atomic Energy Commission, 1951-1952. Present address: Department of Bacteriology, University of Michigan, Ann Arbor, Michigan.

³Predoctoral Fellow of the Life Insurance Medical Research Fund, 1953-1954. Present address: Rackham Arthritis Research Unit, University of Michigan, Ann Arbor, Michigan.

present the endogenous respiration. Blumenthal, Koffler, and Goldschmidt ('52) indicated that the manner in which the cells are made radioactive affects the results obtained with them, and also pointed to the advantage of kinetic over "end-point" data.

The present report is a continuation of that work, and examines the usefulness of various methods. It also introduces the use of unlabeled cells (C^{12} -cells) respiring on uniformly-labeled substrates ($U\text{-}C^{14}$ -substrates) as a general method for following the course of the endogenous respiration. This technique is based on the fact that endogenously produced CO_2 from C^{12} -cells results in the dilution of the $C^{14}O_2$ derived from a radioactive substrate. Moreover, the values for substrate oxidation and assimilation (as determined by the distribution of C^{14} between the CO_2 and the cells) can be compared to such values calculated from manometric data on the basis that the endogenous metabolism proceeds either in an undisturbed manner or is inhibited.

METHODS

Preparation of cells. Vegetative cells of *Penicillium chrysogenum*, strain Q176, were grown in submerged culture from a spore inoculum at $25^\circ C$ in a medium containing either glucose (Stout and Koffler, '51) or acetate (Goldschmidt, Yall, and Koffler, '56) as the main source of carbon.

"Incompletely- C^{14} -labeled" cells (Inc- C^{14} -cells) were prepared from cells grown for 40 to 42 hours in a medium containing non-radioactive acetate or glucose. The cells were harvested on a Buchner funnel, washed with M/15 phosphate buffer (pH 6), and then resuspended in media containing $U\text{-}C^{14}$ -glucose or a mixture of 1- C^{14} - and 2- C^{14} -acetate. Growth continued in the radioactive medium (C^{14} -medium), under agitation by a reciprocating shaking machine (88 four-inch strokes per minute), in a closed system similar to the one described by Martin and Wilson ('51). Air was circulated by means of a small diaphragm pump. After growth on the C^{14} -medium for 6 hours, the cells were harvested.

“Uniformly- C^{14} -labeled” cells (U- C^{14} -cells) were grown from spores in the appropriate C^{14} -medium. Since these cells usually were grown for ca. 45 hours, the air in the closed system was replaced aseptically at appropriate intervals. The cells were harvested and washed in the same manner as the Inc.- C^{14} -cells.

“Starved cells” were prepared by allowing freshly-harvested cells, suspended in M/15 phosphate buffer (pH 6), to respire endogenously on a reciprocating shaking machine for two hours at 25°C.

Manometric procedures. Oxygen consumption and CO_2 evolution were measured by the techniques described by Umbreit, Burris, and Stauffer ('49) at pH 6 (corrected for dissolved CO_2) and 30°C in air. Substrate concentrations are expressed as final concentrations.

Techniques involving C^{14} . $C^{14}O_2$ was trapped in 20% NaOH in the center well of Warburg flasks (no filter paper wick was used in the center well).

During experiments in which the assimilation of U- C^{14} -substrates was measured, Warburg flasks contained 2.0 ml of a cell suspension (2–4 mg dry wt/ml) in the main compartment, 0.5 ml of a U- C^{14} -substrate solution in one side arm, and 0.5 ml of 70–72% perchloric acid in the other side arm. The substrate was tipped into the main compartment at 0 time, and O_2 consumption was measured at 10 minute intervals. The experiment was stopped by the addition of the perchloric acid when the substrate was depleted (i.e., when the rate of the total respiration equaled the rate of the endogenous respiration). The flasks were shaken for at least one additional hour to allow for complete absorption of the CO_2 . When acid was added to one set of flasks, another set of duplicate flasks was removed, and the cell suspensions contained in them were withdrawn. The cells were separated from the liquid by centrifugation, and were washed twice with distilled water. The supernatant liquid and washings were combined, acidified with *N* HCl, evaporated to dryness *in vacuo*, and combusted (Blumenthal *et al.*, '52). The harvested cells were killed by

heat (100°C, 10 min.), transferred to oxidation flasks, dried, and combusted. The alkali in the center wells of the flasks to which acid had been added was removed. BaCl_2 was added to precipitate the carbonate. The BaCO_3 formed was collected by centrifugation, washed twice with 95% ethanol, plated on microporous porcelain filter discs, and counted. The specific activity of the CO_2 released from C^{12} -cells during the oxidation of a given amount of U- C^{14} -substrate was calculated on the basis of the total CO_2 released in additional duplicate flasks without alkali in the center wells, and also on the basis of the total CO_2 minus the CO_2 released endogenously in control flasks.

To measure the rate at which C^{14}O_2 was released from U- C^{14} -cells, 2 ml aliquots of washed labeled cells suspended in buffer were placed in duplicate Warburg flasks containing 0.2 ml NaOH in the center well, 0.5 ml substrate or buffer in one side arm, and 0.5 ml 70–72% perchloric acid in the other. The experiment was started by the addition of substrate or buffer to the main compartment, and stopped at desired intervals of time by the addition of perchloric acid. One hour or more after the addition of the acid, the C^{14}O_2 trapped by the alkali in the center wells was removed and prepared for plating and counting as indicated above.

Organic materials were oxidized in the apparatus of Stutz and Burris ('51) with the Van Slyke-Folch reagent ('40). The radioactivity on the disc was counted with either a thin end-window Geiger-Muller tube or a windowless gas-flow counter with an accuracy of $\pm 5\%$. The counts were corrected, by a graphical method, to the activity at zero self-absorption (Schweitzer and Stein, '50).

Materials. U- C^{14} -glucose was obtained from the Nuclear Instrument and Chemical Co., Chicago, Ill., and the Isotopes Division, Oak Ridge National Laboratory, Oak Ridge, Tenn. The 1- C^{14} - and 2- C^{14} -acetate were obtained from Tracerlab, Boston, Mass.

EXPERIMENTAL

When washed suspensions of microorganisms oxidize glucose or other substrates in the absence of nitrogenous compounds, the amount of O_2 utilized, or CO_2 evolved, by the time the substrate has disappeared completely from the medium is usually less than the theoretical amount for the complete oxidation of the substrate. This phenomenon was

TABLE 1

The percent of theoretical O_2 uptake by glucose-grown cells metabolizing glucose or acetate

| SUBSTRATE ADDED (μM) | TIME OF SUBSTRATE EXHAUST. (MIN.) | % THEOR. O ₂ UPTAKE | |
|--------------------------------|--|--------------------------------|------------------------------|
| | | Uncorr. for endog. resp. | Corr. for endog. resp. |
| Glucose | | | |
| 3 | 50 | 47.2 | 12.0 |
| 6 | 60 | 30.4 | 12.8 |
| 9 | 70 | 31.4 | 13.1 |
| 15 | 150 | 32.8 | 17.8 |
| | | (47.2 — 30.4 = 16.8)* | (17.8 — 12.0 = 5.8)* |
| Acetate | | | |
| 3 | 50 | 118.0 | 29.0 |
| 6 | 90 | 120.0 | 43.3 |
| 9 | 100 | 91.0 | 34.6 |
| 15 | 140 | 82.7 | 37.4 |
| | | (120.0 — 82.7 = 37.3)* | (43.3 — 29.0 = 14.3)* |

* Maximum difference between values obtained for the various substrate concentrations.

recognized first by Barker ('36), and named "oxidative assimilation." In this process a portion of the substrate is oxidized to CO_2 and water, while the remainder is converted to cellular materials. Such a "limited synthesis" apparently occurs in many microorganisms (Clifton, '46, '52). Barker assumed that relatively constant portions of substrate were oxidized and assimilated, regardless of the concentration of substrate used. By comparing manometric data corrected for the endogenous respiration with those not so corrected,

he then determined which practice was in agreement with that assumption. This method was used in the present investigation (referred to as "manometric method"). Resting cell suspensions of *P. chrysogenum* (previously grown on media containing glucose or acetate as the main source of carbon) were allowed to oxidize 3, 6, 9, or 15 μ moles of glucose or acetate to completion. From the amount of O_2 consumed by

TABLE 2

The percent of theoretical O_2 uptake by acetate-grown cells metabolizing glucose or acetate

| SUBSTRATE ADDED (μM) | TIME OF SUBSTRATE EXHAUST. (MIN.) | % THEOR. O ₂ UPTAKE | |
|--------------------------------|--|--------------------------------|------------------------------|
| | | Uncorr. for endog. resp. | Corr. for endog. resp. |
| Glucose | | | |
| 3 | 70 | 52.2 | 14.8 |
| 6 | 110 | 42.6 | 14.3 |
| 9 | 130 | 38.2 | 16.2 |
| 15 | 220 | 40.5 | 20.2 |
| | | (52.2 — 38.2 = 14.0)* | (20.2 — 14.3 = 5.9)* |
| Acetate | | | |
| 3 | 30 | 82.8 | 32.7 |
| 6 | 50 | 76.8 | 35.9 |
| 9 | 60 | 66.4 | 33.9 |
| 15 | 80 | 60.8 | 35.4 |
| | | (82.8 — 60.8 = 22.0)* | (35.9 — 32.7 = 3.2)* |

* Maximum difference between values obtained for the various substrate concentrations.

the time the substrate was exhausted, and the amount of O_2 theoretically necessary for the complete oxidation of the substrate, the per cent of theoretical O_2 uptake was calculated. These values are more constant for the various concentrations of substrate when the value for the endogenous respiration is subtracted, regardless of whether the cells are grown in a medium containing glucose or acetate as the main source of carbon (tables 1 and 2). In the case of cells oxidizing glucose, this is true only if the values for the oxidation of the lowest

level of glucose also are considered. If these values are neglected, it would be difficult to decide which method of calculating the data gives greater constancy of values. The situation is less ambiguous in the case of acetate oxidation. When 3 or 6 μ moles of acetate are being oxidized by glucose-grown cells, at least part of the endogenous respiration must continue, since the total amount of O_2 consumed in

TABLE 3

The effect of starvation or previous oxidation of acetate on the percent of theoretical oxidation of acetate by acetate-grown cells

| ACETATE ADDED (μM) AFTER PREVIOUS OXIDATION OF μM ACETATE | TIME OF SUBSTRATE EXHAUST. (MIN.) | % THEOR. O_2 UPTAKE | |
|--|--|--------------------------------|------------------------------|
| | | Uncorr. for endog. resp. | Corr. for endog. resp. |
| 0 | | | |
| 6 | 60 | 74.2 | 43.4 |
| 12 | 90 | 64.6 | 42.8 |
| 18 | 110 | 58.6 | 40.8 |
| 24 | 150 | 58.7 | 41.3 |
| 30 | 180 | 57.4 | 41.1 |
| | | (74.2 — 57.4 = 16.8)* | (43.4 — 40.8 = 2.6)* |
| 30 | | | |
| 6 | 60 | 69.0 | 33.0 |
| 12 | 90 | 67.9 | 40.7 |
| 18 | 145 | 69.7 | 41.5 |
| 24 | 140 | 58.7 | 35.0 |
| 30 | 145 | 54.7 | 37.8 |
| | | (69.7 — 54.7 = 15.0)* | (41.5 — 33.0 = 8.5)* |

* Maximum difference between values obtained for the various substrate concentrations.

the presence of substrate exceeds the theoretical amount of O_2 needed for the complete oxidation of the amounts of acetate involved (table 1).

The effect of larger substrate concentrations was examined in an experiment in which 0, 6, 12, 18, 24, or 30 μ moles of acetate were added from the side arms of two sets of duplicate Warburg flasks. The cells in one set of flasks were allowed

to oxidize 30 μ moles of acetate to completion just prior to these additions, while in the other set of flasks the cells were starved for the same length of time. These experimental variations were made to permit observations as to whether pretreatment of the cells (i.e., previous oxidation of the substrate or starvation) affects the information obtained from this type of experiment. The data in table 3 indicate that the use of higher concentrations of acetate (in another experiment up to 60 μ moles was used) does not alter the conclusion that data calculated on the basis that the endogenous respiration continued were more uniform than the values obtained on the assumption that the endogenous respiration had ceased. Furthermore, starvation or previous utilization of acetate in concentrations up to 60 μ moles does not seem to affect the conclusion reached (cf. Levring, '45).

Since the manometric method is subject to some criticism (see discussion), other methods were sought that would give data with which the above results could be compared. The most apparent method involves the use of U- C^{14} -cells. Cells of *P. chrysogenum* were grown from spores in a medium containing U- C^{14} -acetate. The release of $C^{14}O_2$ from such cells is a measure of their endogenous respiration. Figure 1 shows that the release of $C^{14}O_2$ from cells grown on U- C^{14} -glucose continues approximately at the same rate, regardless of whether C^{12} -glucose or C^{12} -acetate is being metabolized at the same time. The endogenous metabolism of cells grown on U- C^{14} -acetate behaves strikingly differently when C^{12} -acetate is the substrate being oxidized, as is shown in figure 2. Acetate inhibits the endogenous metabolism of acetate-grown cells while glucose does not. This finding is in disagreement with the conclusions that one might have reached using the manometric method. The method employing U- C^{14} -cells is a more direct approach towards learning about the fate of the endogenous metabolism than the manometric method and is more likely to give reliable information.

The cells need to be uniformly labeled, if the release of $C^{14}O_2$ is to be a realistic criterion for their endogenous

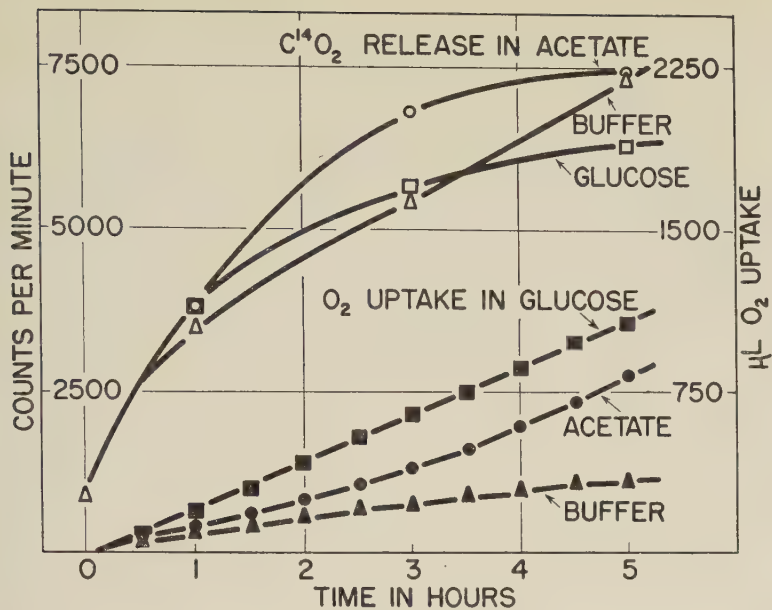


Fig. 1 The effect of 0.1 M glucose or acetate on the time course of O_2 uptake and $C^{14}O_2$ release from cells labeled by growth for 45 hours in a medium containing $U-C^{14}$ -glucose. Manometric readings were made every 10 min., but not all of them are recorded.

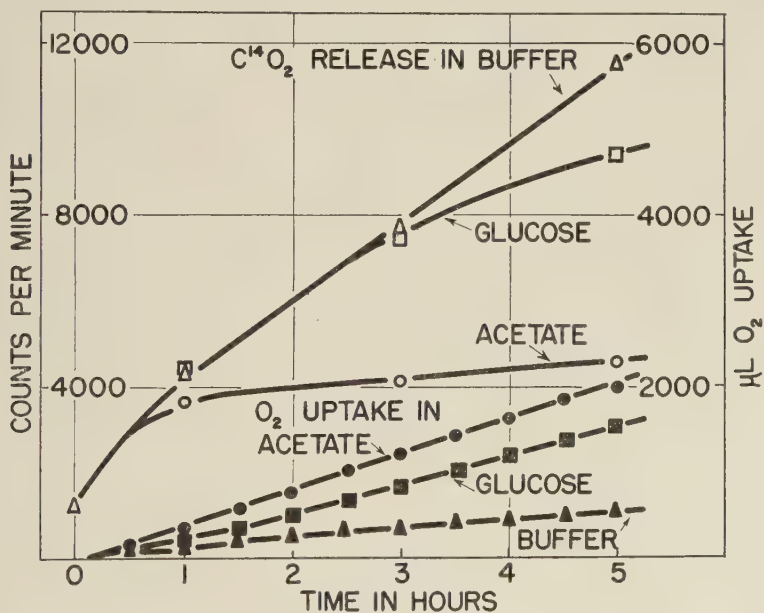


Fig. 2 The effect of 0.1 M glucose or acetate on the time course of O_2 uptake and $C^{14}O_2$ release from cells labeled by growth for 45 hours in a medium containing $U-C^{14}$ -acetate.

metabolism. Inc.-C¹⁴-cells, as is shown in figures 3 and 4, respond quite differently to externally supplied substrates than do U-C¹⁴-cells. This is not astonishing, if one considers that the concurrent oxidation of exogenous substrates may affect the metabolism of different endogenous materials in different ways. With Inc.-C¹⁴-cells one might expect various results, depending upon which of the various cellular components becomes predominantly radioactive during a given labeling procedure.

A satisfactory approach towards measuring the course of the endogenous respiration during exogenous metabolism involves the use of C¹²-cells and U-C¹⁴-substrates. This is illustrated by data in table 4. For example, cells grown in a medium in which C¹²-glucose was the main source of carbon were allowed to oxidize 3 μ moles of U-C¹⁴-glucose. After exhaustion of the substrate, as determined manometrically by the return of the rate of the total respiration to that of the endogenous activity, it was found that 21% of the radioactivity utilized had appeared as CO₂, while 69% was assimilated. Five per cent was in the solution, either as unused glucose or metabolic products of glucose. This was the largest amount of radioactivity that was encountered in the supernatant liquid. The manometric estimation as to the exhaustion of the substrate was fairly reliable in all instances studied, as can be seen by the low percentage of radioactivity left at the end of the experiments so examined. Using the manometric data on the amount of CO₂ liberated or O₂ taken up in the absence and presence of glucose, we calculated the per cent substrate oxidized and that assimilated, on the basis that the endogenous metabolism had continued at its normal rate or had ceased. These estimates were compared to the values as determined by the distribution of the substrate-C¹⁴ between the CO₂ and the cells. For instance, if one assumes that the endogenous respiration had continued, one reaches the conclusion that 23 or 19% (on the basis of data on CO₂ release and O₂ uptake, respectively) of the glucose utilized was oxidized to CO₂; on the assumption that the endogenous

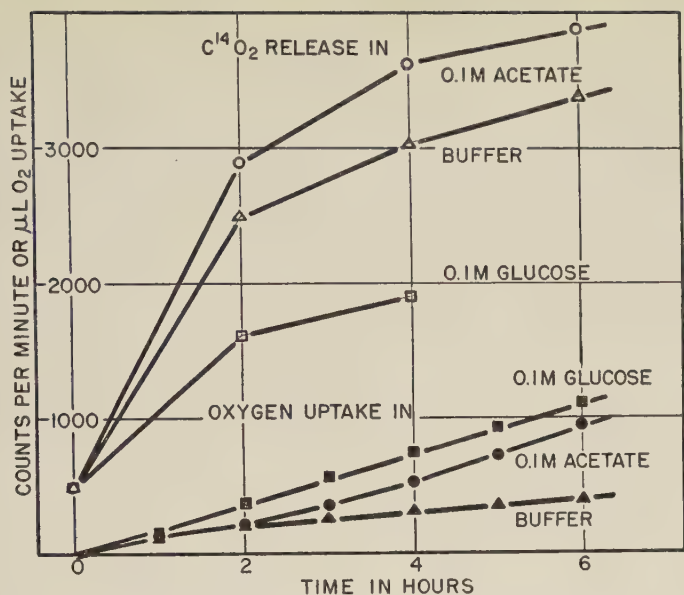


Fig. 3 The effect of 0.1 M glucose or acetate on the time course of O_2 uptake and $C^{14}O_2$ release from cells labeled during growth for 6 hours in a medium containing $U-C^{14}$ -glucose.

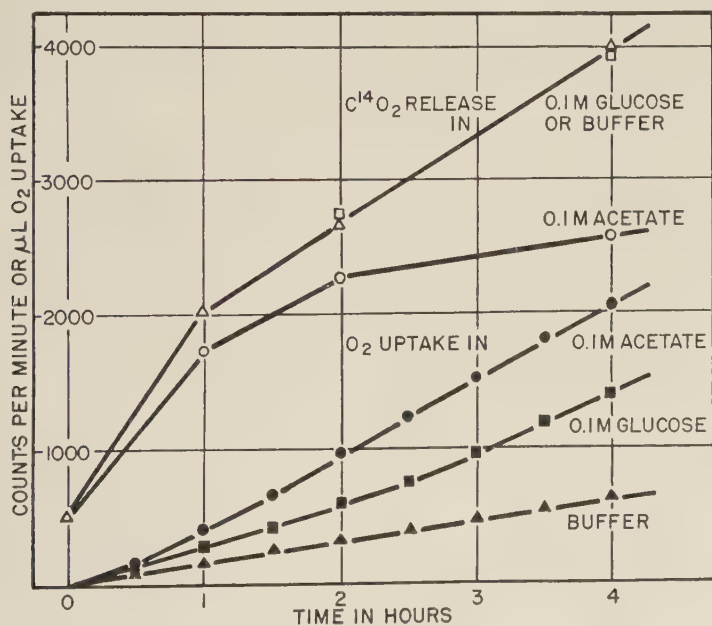


Fig. 4 The effect of 0.1 M glucose or acetate on the time course of O_2 uptake or $C^{14}O_2$ release from cells labeled during growth for 6 hours in a medium containing $U-C^{14}$ -acetate.

TABLE 4

The endogenous respiration of C¹²-glucose-grown and C¹²-acetate-grown cells as affected by concurrent utilization of U-C¹⁴-glucose or U-C¹⁴-acetate

| SUBSTRATE | μM | CPM/ μM C | TIME OF SUBSTRATE EXHAUST. (MIN.) | FRACT. | CPM | % ACT. RECOV. | μM CO ₂ RELEASED | | % SUBSTRATE ACCOUNTED FOR ² | | μM O ₂ CONSUMED | | % SUBSTRATE ACCOUNTED FOR ³ | | SPEC. ACT. CO ₂ (OPM/ μM O) |
|---------------------|---------------|----------------------|--|------------------------------------|-----------------------|------------------|---|--------------------|---|--------------------|--|--------------------|---|--------------------|---|
| | | | | | | | Uncorr. | Corr. ¹ | Uncorr. | Corr. ¹ | Uncorr. | Corr. ¹ | Uncorr. | Corr. ¹ | |
| | | | | | | | | | | | | | | | |
| Glucose-grown cells | | | | | | | | | | | | | | | |
| Glucose | 3 | 340 | 110 | CO ₂ Cells Super. | 1300 4200 320 | 21 69 5 | 9.6 | 4.2 | 53 42 5 | 23 72 5 | 9.1 | 3.5 | 50 45 5 | 19 76 5 | 135 300 |
| | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | |
| Glucose | 6 | 460 | 180 | CO ₂ Cells Super. | 5500 12600 800 | 33 76 5 | 16.2 | 11.7 | 45 51 5 | 32 64 5 | 17.2 | 10.0 | 48 49 5 | 28 67 5 | 340 470 |
| | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | |
| Acetate | 32 | 450 | 320 | CO ₂ Cells Super. | 11400 15750 280 | 40 55 1 | 40.3 | 27.8 | 63 36 1 | 43 56 1 | 48.5 | 30.3 | 76 23 1 | 47 52 1 | 280 410 |
| | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | |
| Acetate-grown cells | | | | | | | | | | | | | | | |
| Glucose | 6 | 550 | 160 | CO ₂ | 3500 | 18 | 16.3 | 7.3 | 45 | 20 | 15.8 | 6.2 | 44 | 17 | 215 480 |
| | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | |
| Acetate | 6 | 980 | 70 | CO ₂ Cells Super. | 6100 6550 160 | 52 56 1 | 7.0 | 3.7 | 58 41 1 | 31 68 1 | 7.3 | 3.8 | 60 39 1 | 32 67 1 | 875 1650 |
| | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | |
| Acetate | 30 | 960 | 255 | CO ₂ Cells Super. | 30200 22850 800 | 53 40 1 | 30.8 | 22.4 | 52 47 1 | 37 62 1 | — | — | — | 980 | 1350 |
| | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | |
| Acetate | 3 | 1670 | 60 | CO ₂ | 4300 | 43 | 5.6 | 2.8 | 94 | 48 | 5.8 | 2.7 | 97 | 45 | 720 1500 |
| | 6 | 835 | 90 | CO ₂ | 4800 | 48 | 8.7 | 4.8 | 73 | 40 | 9.5 | 5.1 | 79 | 42 | 560 1000 |
| | 12 | 420 | 105 | CO ₂ | 5700 | 57 | 11.5 | 7.0 | 49 | 30 | 13.1 | 8.1 | 55 | 34 | 500 800 |
| | 20 | 250 | 150 | CO ₂ | 5500 | 55 | 20.8 | 14.8 | 52 | 37 | 22.0 | 15.2 | 55 | 38 | 270 380 |
| | 30 | 167 | 215 | CO ₂ | 5000 | 50 | 28.8 | 20.0 | 48 | 33 | 32.0 | 22.0 | 53 | 37 | 175 250 |
| Acetate | 30 | 990 | 240 | CO ₂ | 29800 | 50 | 31.4 | 13.3 | 52 | 22 | 36.0 | 17.0 | 60 | 28 | 950 2240 |

¹ Corrected for the endogenously produced CO₂ and O₂.

² Based on amount of CO₂ released.

respiration had been stopped, the calculated value was 53 or 50%. The former values are almost identical to the one actually observed (21%). The CO_2 liberated should have the same specific activity as the carbon atoms of glucose (340 cpm/ $\mu\text{mole C}$), unless the C^{14}O_2 liberated became diluted by C^{12}O_2 from the C^{12} -cells. Since the specific activity of the CO_2 released, as calculated on the basis that the endogenous respiration continued at its normal pace, was 300 cpm/ $\mu\text{mole C}$, the basis of calculation seems to be valid. On the assumption that all of the CO_2 came from C^{14} -substrate, one obtained a value for the specific activity of the C^{14}O_2 considerably below that for the initial substrate. The conclusions reached for the experiments summarized in table 4 were that acetate in all levels used but the lowest (3 μmoles) inhibits the endogenous respiration of acetate-grown cells, while glucose does not; neither glucose nor acetate seems to affect the endogenous respiration of glucose-grown cells. These conclusions are in agreement with those obtained when U- C^{14} -cells and C^{12} -substrate were used, but show a discrepancy with at least one of the conclusions that would have been reached on the basis of the manometric method.

DISCUSSION

Until recently only little progress has been reported concerning the methodology for determining the status of the endogenous metabolism during the simultaneous metabolism of exogenous substrates. For this reason, several approaches were tried in this investigation. From the dilution of the radioactivity in the respiratory CO_2 liberated when unlabeled mycelial pads of *Aspergillus niger* were oxidizing C^{14} -labeled compounds (these observations were incidental to another investigation), Lewis and Weinhouse ('52) concluded that the endogenous respiration of their test organism continued to some extent during the concomitant oxidation of the exogenous substrates used. This type of observation in conjunction with other determinations was adapted in the present study to a general procedure by which the contribution of the endogenous

respiration to the total respiration can be estimated. Several data are essential: (1) The specific activities of the C^{14} -substrate and the $C^{14}O_2$ resulting from its oxidation. If the specific activity of the $C^{14}O_2$ is less than that of the substrate, $C^{12}O_2$ must have been released from the C^{12} -cells. (2) The proportion of the radioactivity found in the CO_2 , the cells, and the supernatant liquid. The first value indicates how much of the substrate was oxidized, the second how much was assimilated, and the third measures either the amount of substrate remaining and/or metabolic products formed; whether the activity in the supernatant liquid represents unused substrate or metabolic product usually can be ascertained by chemical analysis. In almost all of these experiments the amount of substrate remaining at the end of the experiments was relatively small (1-5%). (3) The amount of O_2 consumed and/or CO_2 released by the end of the experiment (which may, but need not be, the time at which the substrate is completely exhausted), and the total endogenous respiration during that period. From the volume of the endogenous respiration one can calculate, using the data of 1, what the dilution of the specific activity of CO_2 would have been, if all or a portion of the endogenous respiration had continued at its normal rate. From the theoretical amount of O_2 necessary to oxidize the substrate completely to CO_2 and water (or from the amount of CO_2 theoretically produced by a given substrate), and the amount of O_2 actually consumed (or the amount of CO_2 actually released), one can calculate how much of the substrate was oxidized and how much probably was assimilated (knowing the amount of substrate having remained unused from the isotope and chemical data). These calculations are made on the basis that the endogenous respiration continued at its original rate, or was partially or completely inhibited. The proportions of substrate that are oxidized and assimilated are known from the isotope data, and one therefore can decide by comparison with the manometric data, which manner of calculation (i.e., using a correction for the endogenous respiration or not) gives comparable values. Agreement between con-

clusions reached from the comparison between the specific activities of the substrate and the respiratory CO_2 , and from manometric data involving O_2 uptake and release of CO_2 constitutes good evidence that the release of CO_2 can be used as a measure of respiration.

Using this technique we concluded that the endogenous respiration of glucose-grown cells is not reduced by the concurrent oxidation of either acetate or glucose. The endogenous respiration of acetate-grown cells, on the other hand, while not affected by glucose is suppressed by acetate in concentrations greater than 3 μmoles ; at 6 μmoles the inhibition of the endogenous metabolism is partial, and in greater concentrations complete. Data obtained when U- C^{14} -cells metabolized C^{12} -substrates verify the essential features of these observations. However, while the former method indicates that the endogenous metabolism of acetate-grown cells must have stopped immediately or soon after the addition of acetate (in inhibitory concentrations), we found with U- C^{14} -cells that they released C^{14}O_2 actively for at least 30 to 45 minutes after the addition of acetate, and slightly even thereafter. Though this discrepancy deals with a detail that does not cause a distortion of perspective, it warranted further investigation. Unfortunately, the reason for this discrepancy so far has eluded analysis.

The validity of the manometric method is subject to question, especially when applied as the sole manner of reaching a decision as to the course of the endogenous metabolism. As mentioned previously, this method is based on the premise that over a wide range of "substrate/cell" ratios a relatively constant proportion of the substrate is oxidized and assimilated. There are only a few data available regarding this assumption. Stout and Koffler ('51), for instance, found with cells of *P. chrysogenum* grown on glucose as the main source of carbon, that the ratio of the "amount of glucose utilized/increase in the dry weight of the cells" was fairly constant over a range of glucose concentrations that varied from 0.005 to 1M. The data in the present paper are not sufficiently ex-

tensive regarding this point to be used without reservation, but it seems that a relatively constant proportion of the substrate appears as CO_2 , regardless of the substrate concentration, except perhaps when the lowest concentration of acetate was used (3 μmoles). In any case, the basic assumption in the manometric method requires the support of a larger body of data. Moreover, as Barker himself noted ('36), at times, values for the amount of assimilation are obtained that are just as constant when corrected for the occurrence of endogenous respiration as when they are not so corrected; thus, a judgment as to the status of the endogenous respiration is at times impossible. At best, only an "either-or" decision can be made. More disconcerting is the discrepancy reported in this paper between results obtained by the manometric method and more direct approaches. On the basis of the manometric method one would have reached the conclusion that the endogenous respiration of acetate-grown cells continued at their normal rate in the presence of acetate, while the direct methods indicated otherwise. The failure of the manometric method to reveal the real situation may be due to the fact that the major premise is not always valid.

There are cases in which the manometric method is reliable, such as when the amount of substrate oxidized, as calculated on the assumption that the endogenous respiration is inhibited completely, exceeds the amount of substrate actually furnished to the organism (cf. table 1, oxidation of acetate by glucose-grown cells). The following workers observed examples of such situations: Gerard and Falk ('31), Winzler ('40), Pickett and Clifton ('43), Anderson ('45), Clifton ('46), Norris, Campbell, and Ney ('49), Vishniac ('49), Stokes ('51), Wilner and Clifton ('54), and Marino and Clifton ('55). Under these circumstances the conclusion that at least part of the endogenous respiration is operative in the presence of substrate can be drawn with confidence. The substrate most frequently found to bring about results of this sort is acetate.

Correcting for the endogenous metabolism by subtracting the volume of O_2 taken up by the cells without exogenous sub-

strate from that taken up by cells with exogenous substrate may give one the impression that the endogenous and exogenous metabolism lead independent existences. This, however, need not be so. When part of an exogenous substrate is assimilated, some of it may be converted to materials that the cell uses endogenously. Most likely there is an interplay between the two, and the question as to when the substrate ceases to be exogenous and becomes endogenous involves an exercise in semantics.

During the experiments in which the release of $C^{14}O_2$ is being measured from U- C^{14} -cells, the $C^{14}O_2$ is representative of the endogenous material before the introduction of the C^{12} -substrate, but it becomes less so as the C^{12} -substrate is assimilated. An eventual drop in the rate at which $C^{14}O_2$ is released from U- C^{14} -cells oxidizing C^{12} -substrates as compared to the rate of release from U- C^{14} -cells in the absence of such substrates, need not be interpreted as indicating an inhibition of the endogenous respiration. The decrease in the release of C^{14} after several hours may reflect a dilution of the radioactive endogenous reserves by newly formed unlabeled endogenous materials. Unless this is kept in mind, this limited synthesis of unlabeled endogenous materials may give rise to errors of interpretation. During active growth cells synthesize all the compounds needed in their molecular economy. The endogenous metabolism of such cells depends upon the conditions under which these cells are grown. This may be so not only because the levels of cellular materials may be different under various growth conditions, but also because the concentrations of various enzymes present in many cases may be determined by the inducing effect of their substrates. Under conditions of limited synthesis (i.e., resting cells in the absence of nitrogen compounds) the character of the endogenous metabolism may change while the organism metabolizes a given substrate, because the endogenous reservoir is not only being replenished by oxidative assimilation, but also altered in composition, depending upon the types of compounds synthesized under these circumstances. Since some of these newly formed endogenous

materials may be more or less readily attacked than the original endogenous reserves, an apparent inhibition of the endogenous metabolism (i.e., metabolism of endogenous substrates present at the beginning of the experiment) may or may not occur. In view of these possibilities, it is difficult to agree with the conclusion of Moses and Syrett ('55) that a decrease in the rate of $C^{14}O_2$ release upon addition of a substrate categorically means that there has been a suppression of the endogenous metabolism. As was mentioned previously (Blumenthal et al., '52), it seems advisable to chart the course of the endogenous metabolism at several intervals of time rather than at the end of a given experiment, because kinetic data may give some clues regarding the pitfalls inherent in the use of U- C^{14} -cells and C^{12} -substrates.

The release of $C^{14}O_2$ from Inc.- C^{14} -cells is not representative of the total endogenous turn-over (Blumenthal et al., '52), because the metabolism of unlabeled or incompletely labeled endogenous compounds is not revealed by such information. Thus it is difficult to assess the results of experiments in which Inc.- C^{14} -cells were used. Reiner and associates ('49), for example, reported that cells of *Saccharomyces cerevisiae* labeled by oxidative assimilation of U- C^{14} -acetate released $C^{14}O_2$ faster during the oxidation of either C^{12} -acetate or C^{12} -glucose than did cells in the absence of these substrates. Similarly, it is difficult to regard the finding of Wiame and Doudoroff ('51) with complete confidence. These workers noted that the oxidation of acetate inhibited the release of $C^{14}O_2$ from cells of *Pseudomonas saccharophila* labeled by oxidative assimilation of C^{14} -acetate. However, in this case there are corroborative data available obtained with a manometric method (Doudoroff, '40; Bernstein, '43) indicating that the endogenous respiration of this organism is inhibited during the oxidation of readily oxidizable substrates. The experience of Gibbs and Wood ('52) illustrates how results can vary with the method used to label the cells. Thus the release of $C^{14}O_2$ was either unaltered or slightly accelerated from U- C^{14} -cells of *Pseudomonas fluorescens* when they oxidized exogenous substrates.

Cells labeled by oxidative assimilation, on the other hand, released radioactive CO_2 significantly faster in the presence of exogenous substrates than in their absence. Essentially similar results were obtained by Moses and Syrett ('55) with *Chlorella vulgaris*. Inc.- C^{14} -cells showed an apparent increase in their endogenous metabolism in the presence of glucose, but this effect was not observed when U- C^{14} -cells were used. Moses and Syrett's data with the Inc.- C^{14} -cells of baker's yeast and *Zygorhyncus moelleri* are difficult to interpret. The results in the present paper indicate that partial growth on C^{14} -substrate is an unsatisfactory method for obtaining U- C^{14} -cells, and not a substitute for growing cultures completely on C^{14} -substrate from small inocula.

The technique of following the course of the endogenous respiration by measuring the C^{14}O_2 released from U- C^{14} -cells has not yet been used widely. Other than furnishing data to show that the endogenous metabolism of acetate-grown cells is inhibited by acetate, as reported in this paper, the information obtained with this technique indicated either no effect on the endogenous respiration or even a stimulation by exogenous substrates (in the case of *Streptomyces coelicolor* (Cochrane and Gibbs, '51), *P. fluorescens* (Gibbs and Wood, '52), *Neurospora crassa* (Heplar and Tatum, '54), *Pasteurella pestis* (Santer and Ajl, '54), and *Chlorella vulgaris* (Moses and Syrett, '55)).

In addition to the methods discussed, there are several others that have been utilized. Some of these are based on indirect evidence. For example, Foster ('44) examined the uptake of O_2 by *Pseudomonas riboflavina* with time, in the presence and absence of ribose. Since the rate of O_2 uptake was constant during the oxidation of ribose and decreased during its absence, he concluded that the endogenous respiration must be inhibited during the oxidation of ribose; otherwise the sum of the two curves would have yielded a resultant rate that decreased with time. This interpretation has been criticized by Hirsch and Wallace ('51) on the grounds that the substrate may have had a stabilizing influence on the cells. Further-

more, an increase in the rate of exogenous respiration with time could have compensated for the decrease in endogenous respiration. In the present work, a comparison of the rates at which U-C¹⁴-cells grown on glucose respire in buffer, acetate, or glucose (fig. 1) shows a situation in which the above reasoning may fail. Although the rate of respiration of the cells without substrate is falling off, the rate increases during the first two hours in the presence of acetate, and remains constant in the presence of glucose. Following the rationale of Foster one might have interpreted these data to mean that the endogenous respiration of this organism is decreased during the oxidation of acetate and glucose. Actually, the isotope data from the same experiment, as well as from others, indicate that no such decrease occurs.

A somewhat analogous type of reasoning was used by Nickerson ('47) in analyzing the data of Bernheim ('42) obtained with *Blastomyces dermatitidis*. Bernheim found an R.Q. of 0.8 for the resting cells and 0.96 upon the addition of glucose. Nickerson believed that the endogenous respiration was suppressed since the R.Q. of 0.8, which is indicative of fatty acid metabolism, increased to 0.96, which is indicative of carbohydrate metabolism. Concurrent participation of endogenous and exogenous activities should have yielded an R.Q. of 0.87. In experiments with *P. chrysogenum* not reported in this paper (Blumenthal and Koffler, unpublished), the R.Q. of resting glucose-grown cells (0.86) was found to change to a value of 1.09 in the presence of glucose. By applying the reasoning of Nickerson, we might have concluded that the endogenous metabolism is inhibited during the oxidation of glucose, whereas all the experimental data in this report indicate that it continues.

A similar type of indirect evidence has been used by Syrett ('51). He reached the conclusion that the endogenous respiration of *Chlorella vulgaris* was inhibited during the oxidation of glucose, because certain concentrations of cyanide stimulated the endogenous respiration while the same concentrations of cyanide did not stimulate the rate of glucose oxidation. Sub-

sequent results with C^{14} -labeled suspensions of this alga can be interpreted differently (Moses and Syrett, '55). Other instances in which the addition of certain substances increased the rate of endogenous metabolism as well as the rate of total respiration have been reported. For example, Borei ('42, '45) found that low concentrations of fluoride increased the endogenous respiration of *Saccharomyces cerevisiae* as well as the total respiration in the presence of an exogenous substrate. Similar results were obtained by Burris and Wilson ('42) with dinitrophenol acting on certain rhizobia, by Stout and Koffler ('51) with azide and dinitrophenol on *P. chrysogenum*, and by Bernheim ('54) with cyanide and azide on a species of *Mycobacterium*. In the absence of other experiments, conclusions from this type of observation should be drawn with reservation.

If an inorganic substrate is being oxidized by a microbial suspension, the endogenous respiration can be followed easily by measuring the release of CO_2 . Thus the finding of Vogler ('42) that the R.Q. of suspensions of *Thiobacillus thiooxidans* remained constant during the oxidation of sulfur can be interpreted to indicate that the endogenous respiration continued unaltered during the oxidation of sulfur.

Using the heavy isotope of O_2 , Brown ('53) concluded that photosynthesis had no effect on the rate of respiration of *Chlorella* suspensions. The endogenous respiration of the blue-green alga, *Anabaena*, on the other hand, was inhibited during photosynthesis at low O_2 tensions (Brown and Webster, '53).

Chemical analyses have been applied in the study of this problem. For example, Fales ('51) examined the carbohydrate fractions of resting suspensions of baker's yeast metabolizing glucose. He found that the endogenous fermentation rate is proportional to the concentration of an alkali-soluble polysaccharide fraction which he believed to be the prime source of energy during endogenous metabolism. Since there was an increase followed by a decrease in this fraction during the course of the metabolism of glucose, it would appear that

the endogenous metabolism of the yeast continued during the concomitant metabolism of glucose. Trevelyan et al. ('52) also applied the use of chemical analyses to this problem. They obtained evidence which suggested that *S. cerevisiae* fermented its endogenous reserves only in the presence of an exogenous substrate. In the absence of other data, conclusions regarding the fate of the total endogenous metabolism based on the chemical analysis of a particular substance should be treated cautiously.

The carbon balance is another type of analysis that has been used to determine the status of the endogenous metabolism during the oxidation of substrates. The value for the amount of carbon assimilated is compared with those values expected from the manometric data corrected or uncorrected for the endogenous respiration. In order to obtain sufficient quantities of cells for accurate carbon determinations, large scale experiments often must be run in parallel with the manometric measurements made in standard Warburg vessels. Thus Wilner and Clifton ('54) found that the values for the amounts of carbon assimilated from glucose by *Bacillus subtilis*, when corrected for the endogenous respiration, agreed closely with those based on manometric data. This indicates that the endogenous respiration is not suppressed in the presence of glucose. In a previous study with *P. chrysogenum*, in which the carbon balance technique was used, it was difficult to decide whether the value for the assimilation of carbon in a large-scale experiment agreed better with the manometric data when a correction for the endogenous respiration was made than when it was not made (Stout and Koffler, '51).

The use of chemical analyses can be extended so that a balance can be constructed which, combined with manometric data, permits calculation of the amount of O_2 that should have been used to cause the disappearance of the substrates. This value is then compared with the value for the actual amount of O_2 utilized, corrected or uncorrected for endogenous O_2 utilization. This technique has been utilized by Ajl ('51, '51a) and Ajl and Wong ('55) with acetate-grown cells of *Escherichia*

coli when acetate, succinate, and α -ketoglutarate were the substrates. Ajl ('51a) calculated that 3650 μ l O_2 should have been utilized to account for the amount of these substrates that had disappeared. Since the exogenous O_2 was 3649 μ l, the endogenous respiration (1327 μ l O_2) must have been completely inhibited. However, this type of experiment is difficult to perform, and is limited to those instances where specific chemical analyses can account for a fairly complete carbon balance.

In certain types of metabolic experiments it is not necessary to know the fate of the total endogenous metabolism in the presence of an exogenous substrate, but only the proportion of a particular metabolic product that is being formed from endogenous materials, and the proportion being formed from the exogenous substrate. Such information can be obtained with U- C^{14} -substrate and C^{12} -cells. After a suitable interval of time, the particular intermediate is isolated and its specific activity is compared with the specific activity of the original material. If the intermediate is derived entirely from the C^{14} -substrate, the metabolic product should have the same specific activity as the substrate. A decrease in the specific activity of the intermediate indicates the endogenous formation of this intermediate or its presence in the cells at the beginning of the experiment. By suitable corrections, the proportion of the intermediate formed during metabolism of the C^{14} -substrate can then be calculated. This procedure has been used to determine, for example, the relative amounts of acetate or ethanol formed from U- C^{14} -glucose, and those present at the start and formed endogenously by the yeast during the course of the experiment (Blumenthal et al., '54).

The results of the present study are of technical interest, because they indicate how respiratory data can be corrected. Of more basic importance is the observation that the previous history of an organism, perhaps by regulating the quantitative distribution of enzymes in the cell, may effect the way in which the endogenous respiration behaves subsequently during the concurrent respiration of exogenous materials. Although the data in the present report are descriptive, and do not offer

support for any specific explanation of the observations made, they do suggest future experiments that may lead to a better understanding of this complex interplay between endogenous and exogenous substrates.

SUMMARY

1. The following methods for determining the course of the endogenous respiration during the concurrent utilization of externally furnished substrates were compared: Barker's indirect manometric method and the more direct methods involving the use of either uniformly- C^{14} -labeled cells and a non-radioactive substrate or unlabeled cells and a uniformly- C^{14} -labeled substrate.

2. The advantages of the last method were emphasized. This approach is based on the fact that endogenously produced CO_2 from unlabeled cells results in the dilution of the $C^{14}O_2$ derived from the radioactive substrate. The participation of the endogenous metabolism in the total respiration can be calculated from the specific activities of the C^{14} -substrate and the $C^{14}O_2$ released, and the total amount of CO_2 liberated endogenously (in the absence of external substrate) during the experimental period. Moreover, the extent of substrate oxidation and assimilation, as determined by the distribution of C^{14} between the CO_2 and cells, can be compared to such values calculated from manometric data. Inasmuch as such calculations are based on the assumption that the endogenous metabolism proceeds in an undisturbed manner or is inhibited to a given extent, the validity of such assumptions can be judged by the observed distribution of the C^{14} .

3. The endogenous respiration of cells of *Penicillium chrysogenum* grown in a medium in which glucose is the main source of carbon is not inhibited by the concurrent oxidation of either acetate or glucose. The endogenous respiration of acetate-grown cells, on the other hand, while not affected by glucose is suppressed by acetate.

4. A comprehensive review of the literature on endogenous metabolism is presented, with a critique of the methodology employed.

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THE SPECTRAL ENERGY CURVE OF LUMINESCENCE OF THE OSTRACOD CRUSTACEAN, CYPRIDINA, AND OTHER LUMINOUS ORGANISMS¹

E. NEWTON HARVEY, A. M. CHASE AND W. D. MCELROY

*From the Marine Biological Laboratory, Woods Hole, Mass., Princeton
University and Johns Hopkins University*

ONE FIGURE

The distribution of energy in any bioluminescence was first determined for the light of the fire-fly by Ives and Coblentz ('09) and again by Coblentz ('12), using a photographic plate to record the spectrum. The resulting plate densities were evaluated as light intensities by calibration with varying durations of exposure to a known constant light intensity.

In 1926, Coblentz and Hughes measured the spectrum of the light emitted from moistened, powdered *Cypridina hilgendorffii* (supplied by one of us, E.N.H.) again using the photographic method. They found a maximum emission peak at 480 m μ with a fairly symmetrical distribution, extending from 410 to approximately 620 m μ .

In 1937, Eymers and van Schowenburg repeated the determination on *Cypridina*. Again the photographic method was used, but the plate calibrations were this time obtained by using a constant duration of exposure and varying the intensity by altering the current through a standardized tungsten filament lamp. They held that varying the intensity and keeping the duration constant was theoretically more correct for calibration purposes than varying the duration and holding the intensity constant, as had been done in Coblentz and Hughes' measurements.

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The curve obtained by Eymers and van Schowenburg was published as spectral energy vs. frequency, and showed two apparent peaks, one at 469 m μ and another at 549 m μ . The latter is probably due to an irregularity in the photosensitive emulsion of the plate, as indicated by the work of Spruit-van der Burg ('50). The curves of both Coblentz and Hughes and Eymers and van Schouwenburg have been reproduced by Harvey ('52, p. 330). This book also contains references to spectral studies on other bioluminescences.

Because of the disagreement in the previous results, we have recently investigated the Cypridina luminescence, using a solution of luciferin first extracted with methanol, then dissolved in water and mixed with luciferase, an aqueous extract of dried Cypridinas. The resulting mixture was perfectly clear and colorless. The concentrations of luciferase and luciferin were so chosen that the light diminished slowly and the spectral distribution could therefore be recorded without marked decrease in intensity. The spectrophotofluorometer of the American Instrument Co. was used to make these measurements.² This instrument is designed for recording fluorescence after excitation at any wave length between 200 and 800 m μ . For our purpose it was used without an exciting lamp and with an ink recorder (Mosely autograph) whose pen moved over the width of the Cypridina spectrum from 400 to 600 m μ , in 4.3 seconds.

The instrument contains two curved gratings and was generally used with three slits of 1/8, 1/16 and 1/32 inch before the gratings and a 3/16 inch slit before the photomultiplier cell of the 1P28 type. The photomultiplier cell picks up chemiluminescent light for each wavelength as a cam moves the gratings. The amplified voltage of the photomultiplier can then be observed on a cathode ray oscillograph or recorded with the Moseley autograph, which we used. Under optimum conditions the record is accurate to ± 5 m μ .

² Kindly loaned for use at the Marine Biological Laboratory at Woods Hole, Mass., by the American Instrument Co.

Figure 1.A shows the spectral distribution for the *Cypridina* luminescence, emitted by luciferin and luciferase in solution in a fused quartz cuvette, as recorded by the instrument: also shown is the same curve corrected for the spectral sensitivity of the average photomultiplier cell of type 1P28. It will be noted that the maximum emission is at 470 m μ and that the curve is not quite symmetrical, with limits around 410 and 620 m μ . Thus all colors of the spectrum are represented in *Cypridina* light but there is no trace of ultraviolet. Our spectrum is undisturbed by color absorption or scattering. With a crude luciferin solution made by extracting dried powered *Cypridina*s with hot water and then cooling, which does have a yellow color, the maximum emission was the same.

For comparison with *Cypridina* luminescence, a clear, pale yellow solution of luminol (aminophthalic hydrazid) plus H₂O₂ was activated with catalyst A (probably an organic cobalt compound) and the luminescence recorded as shown in figure 1.B. The final mixture liberates small bubbles of nitrogen and there is some scattering. It was observed, however, that increased scattering and absorption caused by the addition of a small amount of technical Fuller's earth (mostly clay) of a yellowish gray color to the luminescing mixture shifted the maximum about 10 m μ toward the red. A white super-cel powder did not shift the maximum, even though it made the solution quite turbid.

Three strains of luminous bacteria were available for study, *Achromobacter fischeri* (kept in culture at Johns Hopkins University for many years), and two new bacteria (isolated by Mr. Bernard Weisblum), one from flounder and one from squid at Woods Hole, Mass. All three bacteria gave essentially the same spectral distribution with a maximum at 500 m μ , as indicated in figure 1.C. The bacteria were used in sufficiently low concentration so that the shift in maximum toward the red end, due to scattering and absorption, described by Spruit-van der Burg ('50), was not observed. However, adding yellowish-gray Fuller's earth to the bacteria did shift

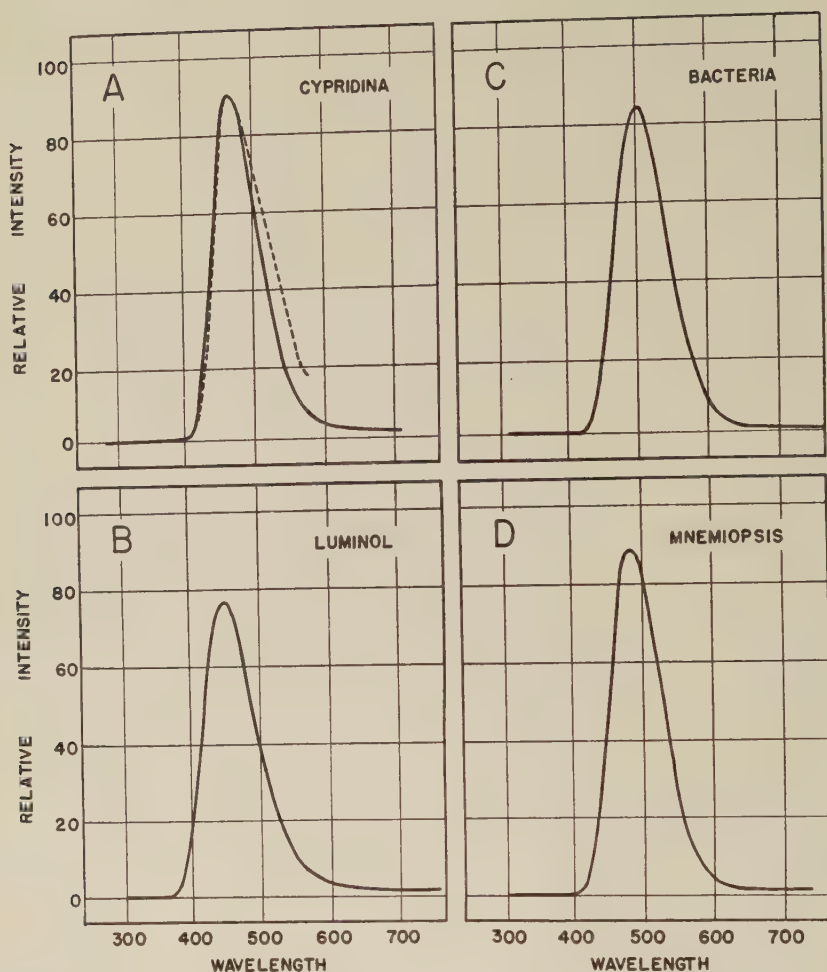


Fig. 1 Spectral energy distribution, in arbitrary units, plotted against wavelength, in millimicrons, for four different luminescences.

A A clear, colorless solution of luciferin and luciferase of *Cypridina hilgendorffii*. The dotted curve shows the data corrected for sensitivity of the photomultiplier tube.

B Luminol (aminophthalic hydrazid) plus hydrogen peroxide, activated by catalyst A. Uncorrected for spectral sensitivity of photomultiplier.

C Luminescence of the luminous bacterium, *Achromobacter fischeri*, uncorrected for photomultiplier sensitivity.

D Luminescence of a ctenophore, *Mnemiopsis leidyi*, squeezed through cheese-cloth, with water then added to produce a luminous tissue suspension. Uncorrected for spectral sensitivity of photomultiplier tube.

the maximum about 20 m μ toward the red, while super-cel, calcium carbonate powder, and pyrex glass wool did not. The white super-cel and calcium carbonate greatly increased the light coming from the suspension of bacteria in the cuvette.

A record was also taken of the spectral energy distribution of the light of protoplasts produced from *Achromobacter fischeri* in the following way by Palmer Rogers. The bacteria were first grown in a Farghaly ('50) nutrient medium with 1% NaCl, 1% peptone and 20% sucrose for 18 hours, and then 1000 units of penicillin per ml were added and growth allowed to continue for 5 additional hours. The resulting protoplasts were without normal cell walls, nearly spherical in shape and about 4 micra in diameter. The emission spectrum of these protoplasts did not differ from that of normal *A. fischeri*, despite the fact that optical characteristics at the surface must have been quite different in the penicillin-treated forms.

Another luminous animal at Woods Hole, the ctenophore, *Mnemiopsis leidyi*, was studied in a rather crude way. If this ctenophore is squeezed through cheesecloth, the resultant "brei" will luminesce from many points of light in the broken tissues of the animal, but the light soon disappears. Luminescence can be evoked from such a suspension by adding distilled water. Points of light appear and disappear, the average light emission of the suspension lasting a minute or two. This material was placed in the cuvette and the record shown in figure 1.D was obtained. The maximum is at 490 m μ . To the eye, *Mnemiopsis* light looks decidedly greenish but the actual measurement indicates a maximum in the blue-green. The eye is, of course, a poor judge of the color maximum of luminescences.

Perhaps the most interesting aspect of the present measurements is the indication of a slight but definite asymmetry in all the curves, which becomes more pronounced when they are corrected for the sensitivity of the photo cell. This asymmetry would be still more apparent if additional corrections were necessary for any decrease in light intensity as the measure-

ments are made. These corrections are zero at the blue end and never more than 6% at the red end. The corrected curves all indicate that the wavelength of maximum emission lies toward the blue rather than in the middle of the spectrum. This asymmetry is to be noted in most of the previous records of spectral energy distribution in bioluminescences, and is undoubtedly real. We believe that the results of the present research give the most accurate picture of the emission spectra of luminous animals thus far published. It is unfortunate that the lack of sharp emission lines or any indication of structure in the spectrum prevents the use of such data for identifying the luminous substances concerned. The emission spectrum of luminol is much like that of *Cypridina* luminescence but it is quite certain that luminol and *Cypridina* luciferin are not the same chemically.

SUMMARY

The spectral energy curves for luminescence of the following organisms or solutions have been recorded automatically by a spectrophotofluorometer. The output of the photomultiplier was recorded automatically for each wavelength.

| | <i>Max.</i> | <i>Limits</i> |
|--|-------------|---------------|
| <i>Cypridina hilgendorffii</i> (clear colorless aqueous solutions of luciferin and luciferase) | 470 | 410-620 |
| <i>Achromobacter fischeri</i> (and two new luminous bacteria) | 500 | 420-610 |
| <i>Mnemiopsis leidyi</i> (squeezed through cheesecloth with water added) | 490 | 420-610 |
| Luminol and H_2O_2 activated by catalyst A | 460 | 380-610 |

In all cases the maximum is nearer the blue than the red end of the spectrum.

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